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EFFECT OF IL-15 ON THE OUTGROWTH OF THE EPSTEIN-BARR VIRUS-INFECTED/IMMORTALIZED PERIPHERAL BLOOD MONONUCLEAR CELLS

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Ce mémoire intitulé:

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RÉSUMÉ

Le virus Epstein-Barr a été décrit pour la première fois par Epstein et al. (Epstein et al., 1964) en 1964. EBV est un virus humain lymphotropique omniprésent de la famille des virus herpès gamma (Roizman et al., 1981). L'IL-15 est une cytokine récemment isolée appartenant à la famille des cytokines à quatres hélices α. L'IL-15 fut isolée des surnageants d'une culture de cellules épithéliales de rein simian CV-1/EBNA en 1994 (Grabstein et al., 1994).

Une étude précédente avait montré que la sécrétion de l'IL-15 était induite suite à l'infection des PBMCs humains par le virus HHV-6, un virus herpès lymphotropique (Flamand et al., 1996). Une autre étude de notre laboratoire par Fawaz et al. (1997) a aussi démontré la sécrétion de l'IL-15 suite à l'infection virale (Fawaz et al., Manuscrit en préparation). Notre étude a visé à étudier l'effet de l'IL-15 sur la réponse immune cellulaire suite à l'infection des PBMCs *in vitro* par le virus Epstein-Barr et l'induction d'immortalisation des PBMCs suite à cette infection dans le but de déterminer le rôle de cette cytokine dans la régulation de la réponse immunitaire à l'infection virale aigue. Nos données montrent que l'ajout de l'IL-15hr exogène à des cellules infectées par EBV *in vitro*, exerçait un effet inhibiteur sur la transformation induite par EBV. De plus, cet effet inhibiteur était médié par la fonction cytolytique d'une population cellulaire T CD3+CD56+ qui est induite spécifiquement par l'IL-15 en présence ou l'absence de l'infection par EBV. De plus, cette population cellulaire exprimait l'antigène CD8 confirmant ainsi son phénotype cytotoxique. L'IL-15 n'avait aucun effet direct sur l'expression de gènes de l'EBV ni sur les lymphocytes B infectés. Ces résultats démontrent l'action antitumorale de l'IL-15, et suggère la possibilité de son utilisation dans de futures stratégies de thérapie immune.

L'analyse de la transformation des PBMCs induite par infection avec EBV *in vitro* par observation microscopique, immunofluorescence de la synthèse de protéines EBNAs (fig.1), ainsi que par l'*immunoblotting* de la protéine LMP-1 (fig.2), avait confirmé que l'incubation des PBMCs infectés par EBV en présence de l'IL-15 résultait en régression de la transformation induite par EBV comparée à des cultures de PBMC infectées en l'absence de l'IL-15. Cet effet inhibiteur spécifique observé fut démontré lors de l'incubation de cellules infectées par EBV en présence de l'IL-15 et l'anti-IL-15 pour une période similaire. De plus, l'observation de cet effet chez les PBMCs de personnes séropositives et séronégatives a indiqué que l'effet observé était mémoire-indépendant. Ceci est en accord avec une étude précédante de notre laboratoire sur l'effet exercé par l'IL-2 sur les cultures cellulaires infectées par EBV (Khyatti et al., 1993).

Les résultats obtenus de l'incubation de la lignée B95-8 en présence ou absence de l'IL-15 ont démontré que l'IL-15 n'avait aucun effet direct sur l'expression des antigènes viraux. L'incubation de B95-8 pour six jours en présence de l'IL-15 n'a pas affecté de façon significative l'expression des EBNAs (résultats non montrés). De plus, les données montrent que l'IL-15 n'agissait pas directement sur les cellules B infectées *in vitro* (fig.4). Cependant, une prolifération significative des cellules B non-infectées ainsi que des cellules infectées fut observée en présence de l'IL-15 quinze jours après infection, par comparaison au control.

L'élimination de la transformation induite par EBV des cultures de PBMC de deux semaines après l'ajout de l'IL-15 pour dix jours, suggère que l'IL-15 peut contrôler l'immortalisation des lymphocytes B à une phase tardive de l'infection aussi (fig.3). Dans le but de déterminer le phénotype des cellules responsables de médier l'effet inhibiteur de l'IL-15, des études par

FACS ont été faites, et ont montré qu'en effet une population T CD3+CD56+ proliférait significativement après 21 jours de traitement des PBMCs avec l'IL-15 en présence ou absence d'infection avec EBV (fig.5, a). De plus, des résultats similaires ont été obtenus de donneurs séropositifs et séronégatifs. Ceci nous a amené à deux conclusions importantes: 1) la capacité de l'IL-15 d'induire l'expansion de la population T CD3+CD56+ des PBMCs en l'absence d'infection virale indique l'absence de restriction par le CMH, 2) l'augmentation de cette population chez les PBMCs de personnes séronégatives indique que le mécanisme d'induction est mémoire-indépendant. De plus, nous montrons que l'effet prolifératif induit par l'IL-15 sur les cellules T CD3+CD56+ est accompagné par une diminution significative des cellules T CD3+CD56- pour une période similaire d'incubation (fig.6). Les résultats obtenus par FACS (fig.5, b) montrent l'absence de prolifération de cellules NK CD3-CD16+ de PBMCs préincubés avec l'IL-15 en présence ou absence de l'infection avec EBV pour 21 jours.

En résumé, nos résultats montrent l'importance du rôle de l'IL-15 dans l'activation d'une réponse cellulaire non-spécifique suite à l'infection virale. On a démontré que l'IL-15 induisait spécifiquement et significativement l'expansion d'une population T CD3+CD56+ de manière non-restreinte par le CMH, et que cette population cellulaire exprimait l'antigène CD8 (fig.7). Les résultats démontrent aussi que ces cellules T cytotoxiques pouvaient éliminer complètement la transformation des PBMCs *in vitro* induite par infection avec EBV. Ces données suggèrent un rôle antitumoral de l'IL-15 et proposent son utilisation dans de possibles stratégies thérapeutiques immunes.

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A comparative study of the upregulation of natural killer cytotoxic activity via IL-15 induction by different viruses.

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
AIDS	acquired immunodeficiency syndrome
BL	Burkitt's lymphoma
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
EA	Epstein-Barr virus early antigen
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
gp	glycoprotein
HD	Hodgkin's disease
HHV-6	human herpes virus 6
HIV	human immunodeficiency virus
IFN-α	interferon α
IFN-γ	interferon γ
Ig	immunoglobulin
IL	interleukin
IL-15R	interleukin-15 receptor
IL-2R	interleukin-2 receptor
IM	infectious mononucleosis
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LP	leader protein
LYDMA	lymphocyte-detected membrane antigen
MHC	major histocompatibility complex
NK	natural killer
NPC	nasopharyngeal carcinoma
PBMC	peripheral blood mononuclear cell

PBS	phosphate buffer saline
PCR	polymerase chain reaction
PTLD	Post-transplantation lymphoproliferative disorder
rhIL-15	recombinant human interleukine-15
SDS-PAGE	sodium-dodecyl sulfate polyacrylamide gel electrophoresis
VCA	viral capsid antigen of the Epstein-Barr virus
XLPS	X-linked lymphoproliferative syndrome

SUMMARY

Epstein-Barr virus is a ubiquitous gamma herpes virus that preferentially infects B-lymphocytes and induces immortalization, giving rise to long-term lymphoblastoid B-cell lines (LCL) (Kieff et al., 1985).

The importance of cytokines in the activation of the immune system against invading organism is well documented. IL-15 is a recently recognized cytokine belonging to the four α-helix bundle cytokine family. Originally, IL-15 was isolated from the supernatants of the simian kidney epithelial cell line CV-1/EBNA in 1994 (Grabstein et al., 1994). Although IL-15 shares many biological activities with IL-2, there are several properties of IL-15 distinct from those of IL-2. These differences between IL-2 and IL-15 suggest unique *in vivo* roles for IL-15.

Two recent studies from our laboratory confirmed the induction of IL-15 in the supernatant of PBMCs following viral infection in vitro (Flamand et al., 1996; Fawaz et al., manuscript in preparation). Therefore, the present study was undertaken to investigate the proliferative effect of IL-15 in long term in vitro incubation with PBMC and to study the immunological function of this cytokine following EBV infection.

Results obtained from this study showed for the first time that EBV-infected/transformed cells were completely eliminated in the presence of IL-15 in the culture. IL-15 was seen to exhibit no direct inhibitory effect on neither the EBV genome nor on B cell growth. Indeed, results gathered from proliferation assays showed that IL-15 induced the proliferation of purified B cells. This proliferative effect was seen in the presence or absence of EBV infection. Since IL-15 had no direct inhibitory effect on EBV-induced

transformation of PBMC, and newly established (lymphoblastoid cell lines) LCL were found previously to be relatively resistant to lysis by NK cells (Seeley et al., 1981), we suggested that cellular effectors other than NK cells might be involved in the cellular immune response to EBV infection.

A rapid and significant cellular proliferation occurred as early as 5 days following the addition of IL-15 to the cultured cells. Using several combinations of MoAbs and flow cytometry, the proliferated cells were found to be CD3+CD56+ T cells. The specificity of IL-15 proliferative effect on CD3+CD56+ T cells was ascertained through abrogation of the proliferation when a MoAb against IL-15 was added to the cultures. In addition, we found that the proliferative effect induced by IL-15 on the CD3+CD56+ T cells was accompanied with a significant decrease in the CD3+CD56- T cell subset following a similar period of incubation. Moreover, FACS analysis performed on proliferated CD3+CD56+ T cells showed that the majority of the proliferated CD56+ T cells coexpressed CD8 a characteristic surface antigen of cytotoxic T lymphocytes.

Regarding NK cells and IL-15, as mentioned earlier, studies from our laboratory showed an upregulation of NK activity following viral infection of PBMC in vitro. Data gathered from these studies indicated that IL-15 was specifically responsible for the upregulation of NK activity. Nevertheless, non-of these studies analyzed the prolefirative effect of IL-15 on NK cell population. Data gathered from FACS analysis in the presented study demonstrate the absence of a significant proliferative effect of IL-15 on CD3-CD16+ NK cell subset following incubation of PBMC in the presence of this cytokine for 21 days. Interestingly, we observed a restoration of CD3-CD16+ NK cells within 21 days of incubation with IL-15.

Regarding CD3+CD56+ T lymphocytes, a prior study has indicated

that these cells can be expanded by culturing PBMC in the presence of IFN-γ on day 1, and in the presence of rIL-2, rIL-1α, and a MoAb against CD3 the following day (Schmidt-Wolf et al. 1991). However, we showed that CD3+CD56+ T cells rapidly proliferated following incubation of normal human PBMC in the presence of IL-15 alone starting from the fifth day of culture.

Taken together, these findings ascertain the role of IL-15 in the induction of non-specific immune response that might be mediated by the CD3+CD56+ subset of cells, which in turn would inhibits the outgrowth of the EBV-infected/immortalized cells. Therefore, IL-15 would be a suitable candidate for the immunotherapy of cancer.

DEDICATION

This work is dedicated to:

My beloved father and mother

My brothers AHMAD and MOHAMMAD

My sisters ANWAR and ZAHRA

My best friend for ever

LAMA M. FAWAZ

And finally to my first research director and friend

Dr. GEORGE F. ARAJ

I. INTRODUCTION

CHAPTER 1

Epstein-Barr virus

1.1 History

The Epstein-Barr Virus (EBV) story starts from east Africa where Dr Denis Burkitt was working as a missionary doctor in 1958. He was interested in a novel childhood tumor that he found to be unusually common throughout equatorial Africa (Burkitt et al., 1958). The novel tumor was known later as Burkitt`s lymphoma (BL). Burkitt and based on the climatic factors present in that area, postulated an involvement of an infectious agent in the pathogenesis of BL. In the early 60s, Dr. Burkitt and Dr. Epstein failed to screen viral particles from freshly excised BL tumor biopsies using electron microscopy. In 1964, Epstein and his student succeeded to isolate continuous cell lines from Burkitt`s lymphoma cells (Epstein and Barr, 1964a). When the cultured cell lines where examined, herpes-like particles were seen under electron microscopy (Epstein et al., 1964b). Additional studies revealed that the virus present in BL cells was distinct from other members of Herpesviruses (Epstein et al., 1965). In 1966, Henle et al identified this virus as a new species of the herpesvirus family (Henle and Henle, 1966), and in 1968, Diehl et al found, while following up the seroconversion of a laboratory technician, that this new virus was the causative agent of infectious mononucleosis (IM) (Henle et al., 1968). After its recognition, the virus was constantly detected in neoplastic cells of endemic Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma, raising the question of an etiological role for this virus in the development of these tumors (Zur Hausen et al., 1970). Since then, EBV has been subjected to intensive investigations, and over the last years, and due to

the availability of a variety of sensitive detection techniques and the increased incidence of EBV-associated lymphoproliferative disorders in various forms of immunodeficiency, EBV has been linked to a growing list of human malignancies (kieff, 1996).

1.2 Classification

In the last 50 years, around 100 distinct herpesviruses have been isolated from a wide variety of animal species. These viruses belong to the Herpesviridae family. Herpesviruses have been classified on the basis of biological properties, immunological specificity of their virions, features of virus replication, and certain aspects of genome structure like base composition and gene rearrangement. With respect to biological properties, the herpesviruses have been classified into three subfamilies: Alpha-, Beta-, and Gammaherpesvirinae on their host range, duration of reproductive cycle, cytopathology, and characteristics of latent infection (Roizman et al., 1981). EBV belongs to the gammaherpesvirinae subfamily in which members are characterized by their lymphotropic tropism in vitro. In vivo, viral infection is frequently either at a latent or a lytic stage (Chee and Barrell, 1990).

It is worth noting that at least two EBV types have been identified in most human populations (Sixbey et al., 1989). These EBV subtypes are designated as EBV-1 and EBV-2. Both EBV subtypes show extensive homology except for the genes that encode nuclear antigens (EBNAs) and small polyadenylated RNAs (EBERs) in latently infected cells (Allday et al., 1989). Earlier studies have shown that while both subtypes can be detected from African isolates (Zimber et al., 1986), EBV-1 is more commonly isolated from individuals in western societies. It was suggested that the failure to isolate EBV-2 from PBMC of healthy individuals from western societies

might reflect less aggressive growth of this subtype in blood lymphocytes (Rickinson et al., 1987).

1.3 Viral structure and genome organization

A typical herpesvirion consists of a core containing a linear double stranded DNA, an icosadeltahedral capsid approximately 100-110 nm in diameter containing 162 capsomers with a hole running down the long axis (Wildy and Watson, 1963), an amorphous material, designated as tegument that surrounds the capsid, and an envelope containing viral glycoprotein spikes on its surface (Dolyniuk et al., 1976).

EBV was the first herpes virus whose genome was completely cloned and sequenced (Dambaugh et al., 1980; Paker et al., 1990). The EBV genome consists of a linear double-stranded DNA about 170 kilobases in length (Paritchett et al., 1975). Upon infection, the viral DNA is transported into the nucleus, where it exists predominantly as an extrachromosomal circular molecule termed episome (Yates et al., 1985). The circularization is mediated by the joining of terminal repeat (TR) sequences (Given et al., 1979). The episome can be subdivided into four regions: the short and long unique regions (US and UL, respectively), separated by the major internal repeat (IR) and the TR. The major internal repeat itself is subdivided into several repetitive elements (IR2, IR3, and IR4).

Although the EBV genome contains over a 100 reading frames that potentially encode for as many polypeptides, only a relatively small proportion of these proteins which are expressed either during latent or lytic viral state have been characterized.

1.4 Stage of infection

There are two distinct stages of the EBV life cycle. A productive replicative infection (lytic cycle) in which mature infectious virus particles are assembled and released, resulting in cell death, and a nonproductive infection (latent cycle) in which the virus is incorporated into and replicates with the host DNA, but remains in the latent state i.e., no mature virus is produced (Epstein and Achong, 1979).

1.4.1. Latent infection

EBV infects and enters susceptible cells by interaction of its major viral envelope glycoprotein gp 350 with specific surface receptor which is also reactive with the C3d fragment of the third component of complement, the complement receptor CR2 (also designated as CD21) (Jondal et al., 1976). The interaction between gp 350 and CR2 leads to receptor-mediated endocytosis of the virus (Nemerow and Cooper, 1984). Indeed, until recently it was believed that the EBV receptor is unique to cells of B-lymphoid origin (Ross and Medof, 1985). However, this receptor was recently found to be expressed on several type of human cells including: immature thymocytes (Watry et al., 1991), follicular dendritic cells of tonsils and lymph nodes (Reynes et al., 1985), pharyngeal (Young et al., 1986) and cervical (Sixbey et al., 1987) epithelia. Nevertheless, among haemopoietic cells, the B cell still stands alone in being able to bind and internalize EBV with high efficiency. In vitro, EBV has the ability to infect B-lymphocytes efficiently resulting in persistent latent infection and immortalizaton. Similarly, EBV infects B cells in vivo and produces B lymphoblasts, which also grow indefinitely when

cultured in vitro. The consequences of EBV infection of B-lymphocytes were extensively studied. EBV infection induces RNA and DNA synthesis, expression of B-cell activation markers, cellular adhesion molecules, immunoglobulin secretion, and cell division (Kintner and Sugden, 1981; Amen et al., 1986; Gordon et al., 1984).

Transformation of B cells is a complex process requiring the function of several EBV gene products that serve together to maintain the viral genome in the cell, transactivate viral and cellular gene products important for B-cell growth, interact with B-cell proteins, and prevent B-cell death. These products where mapped to a limited subset of genes, the latent genes, which are coordinately, expressed in the latently infected lymphoblastoid cell lines (LCLs) that arise following viral infection of resting B cells in vitro. Most of the details of latent infection in B-lymphocytes have been drawn from studying EBV gene expression in LCLs. These genes encode at least 11 latent proteins including six nuclear antigens (EBNA1, 2, 3A, 3B, 3C, -leader protein [LP], three integral membrane proteins (LMP1, 2A, 2B) and two small nonpolyadenylated nuclear RNAs (EBER1 and 2) (Kieff, 1996). These viral proteins maintain the latent infection and cause the previously resting B-lymphocytes to continuously proliferate. Alfieri et al (1991) showed that EBNA2 and EBNA5 are the first nuclear antigens expressed following EBV infection of B-lymphocytes (Alfieri et al., 1991). EBNA2 specifically trans-activates CD23, CD21, and c-fgr as well as the LMP promoters (Abbot et al., 1990; Wang et al., 1990; Knutson, 1992). EBNA1, the only EBNA protein that associates with chromosomes during replication (Petti et al., 1990), binds to ori P and enables the EBV episome to be maintained in the nucleus and replicate during the S phase. Due to the similarity between EBNA3A, EBNA3B, and EBNA3C, these proteins are likely to have similar roles in latent infection. EBNA3C protein has been found to up-regulate CD21 mRNA and protein but had no effect on CD23 expression (Wang et al., 1990).

Sample et al (1990) found that latently infected cells have almost 10 times as much LMP1 mRNA as EBNA mRNA, indicating that LMP1 mRNA is much more stable than EBNA mRNA (Sample and Kieff, 1990). LMP1 acts as a direct oncogene in transformation assays (Kieff and Liebowitz, 1990), and expression of LMP1 in epithelial cells transforms them morphologically (Fahraeus et al., 1990). In addition, LMP1 induces many of the changes associated with EBV infection of B-lymphocytes including cell clumping, increased cell surface expression such as CD23, CD40, and class II major histocompatibility complex (MHC-II), and increased expression of the cell adhesion molecules such as LFA1, ICAM1, and LFA3 (Wang et al., 1987). Importantly, LMP1 appears to be expressed in most latently infected cells in vivo and in vitro. LMP1 and EBNA1 are the only latent infection-associated genes that are also transcribed in lytic EBV infection.

LMP2A appears to be expressed in most latently infected cells in vivo and in vitro (Longnecker AND Kieff, 1990). Using mutant recombinant EBV, it was noticed that LMP2A and 2B have no critical effect on either EBV-induced transformation of primary B-lymphocytes nor on the lytic infection that was induced following the treatment of latently infected lymphocytes with inducer of lytic infection (Kim et al., 1993). Two latent gene products, the EBERs are the most abundant EBV RNAs in latently infected cells (Arrand and Rymo, 1982). Similar to LMP2A and 2B, EBERs were noticed to have no difference on the growth of the EBV-infected initiated with EBER-mutant virus. Additionally, no major effects were detected on the permissivity of transformed cells for lytic infection following induction (Swaminathan et al., 1991). Other genes were also found to be expressed during the latent cycle such as BHRF1 (Austin et al., 1988) and BARF0 (Brooks et al., 1993). However, no proteins were detected in typical latent infected cells.

Based on the viral latant gene expression in EBV-associated malignant cells, three major forms of latency have been distinguished. These include latency I as in Burkitt's lymphoma, Latency II as in nasopharyngeal carcinoma, and Latency III as in lymphoblastoid cell lines (LCL).

1.4.2 Lytic infection

The virus ensures its survival in the host infectable target cells, by induction of a lytic cycle, which is tightly controlled. In fact, EBV replication is strongly down regulated in B-lymphoblastoid cells as well as in less differentiated epithelial cells. Only terminally differentiated epithelial cells seem to be capable of efficiently supporting the EBV lytic cycle (wilmes et al, 1981). One of the main differences between latent and lytic infection is the number of proteins that are expressed in the infected cells (Biggin et al., 1987). Entrance of EBV into the lytic state with production of infectious virions is associated with a broad expression of viral proteins, which include enzymes involved in nucleic acid metabolism, and formation of viral capsid proteins. These proteins are classified into early and late antigens based on the time of their expression in the lytic infection. Early antigens are those protein that appear early after the induction of lytic cycle independently of new protein synthesis and and classified into immediate early and delayed early antigen (Henle et al., 1970). Early antigens include a series of polypeptides. The key immediate-early protein is ZEBRA, the product of the BamHI-Z fragment leftward reading frame (BZLF)-1. ZEBRA is a DNA-binding protein that functions as a transactivator of the EBV lytic gene expression and facilitates the transition from latent to lytic infection (Sinclair et al., 1992). Two other important lytic viral early proteins are BCRF1 which is identical to interleukine 10 (IL-10), an immunoregulatory cytokine (Vieira et al., 1991), and BHRF1 that is similar to bcl-2, an inhibitor of apoptosis. The homology of

these early viral proteins with cellular proteins has lead to the speculation of a similar function in EBV-infected cells (Oudejans et al., 1995). EBV early products can be differentiated from late gene products by their resistance to the inhibitors of viral DNA synthesis.

EBV late antigens are mostly structural viral proteins that modify the infected cells so as to permit successful packaging. Late genes with enzymatic or regulatory functions have not yet been defined. The viral late glycoprotein genes are of special interest due to their potential importance in antibody-mediated immunity to viral infection. The known EBV late genes and their respective glycoproteins are: RLLF1 (gp 350/220), BALF4 (gp 110), BXLF2 (gp 85), ILF2 (gp 35/80), and BDLF3 (gp 42) (Beisel et al., 1985). As mentioned previously, gp 350/220 mediates virus binding to the B-lymphocyte receptor, CR2. Moreover, this glycoprotein is a target for most of the human EBV neutralizing antibody response (Thorley-Lawson and poodry, 1982).

New strategies for manipulating the transition to lytic infection have facilitated EBV molecular genetics and biochemical research. Lytic infection can be induced in vitro using chemical inducers or superinfection of cells permissive for lytic virus gene expression. In terms of chemical inducers, phorbol esters, calcium ionophore and cross-linking surface immunoglobulins (sIg) have been widely used (Flemington and Speck, 1990; Takada, 1984). Nevertheless, phorbol esters are among the most reproducible and most broadly used inducers (Laux et al., 1988). A second approach to study viral lytic infection is to induce lytic replication by superinfection of Raji cells with a defective EBV from the P3HR1 cell line (Biggin et al. 1987). It is worth to note that P3HR1 superinfection tends to be more rapid and synchronous in inducing productive infection than chemical inducers. However, In both approaches, the use of chemical inducers or superinfection, maximal late virus protein expression and enveloped virus release required 48 to 72 hours.

1.5 EBV infection

Most humans are infected with EBV early in childhood, but in regions with higher socioeconomic standing and lower population density, infection may be delayed until adolescence or later. The intimate contact facilitates the spread of the viral infection. The virus is spread predominantly via exchange of saliva and usually a pleasant event, kissing, is the best-established route of infection (Straus and Fleisher, 1989). Transmission of the infection through transplantation and blood transfusion are likely and possibly by sexual intercourse, due to the demonstration of EBV in vaginal fluid (Sixbey et al., 1986), however, this was not well documented.

1.5.1 Primary infection

Although nearly 9000 publications have been written since its discovery, many questions concerning the EBV pathogenesis remain unanswered. Available data about the function and infection pattern of EBV provide a compiling evidence for its participation in several diseases. However, our understanding of the events of primary EBV infection is still quite limited since in most populations primary infection occurs within the first 3 years of life (Lang et al., 1977), and is almost asymptomatic.

Most of what is known about primary EBV infection is derived from the study of infectious mononucleosis (IM), the paradigmatic disease that is associated with EBV infection. The term infectious mononucleosis was first utilized by Sprut and Evans in 1920 (Sprut and Evans, 1920), and in 1968, EBV was identified as the causative agent of this disease (Henle et al., 1968).

Infectious mononucleosis, a self-limited lymphoproliferative illness, occurs at all ages, but most cases occur during adolescence and early adulthood (Straus and Fleisher, 1989).

In developing countries, EBV infection occurs early in life. Nearly the entire population of developing countries becomes infected before adolescence. Thus, symptomatic infectious mononucleosis is uncommon in such areas (Straus and Fleisher, 1989). In contrast to developing areas, IM is more prevalent in developed countries. In the areas with high standards of hygiene, such as the United States and Western Europe, EBV infection may be delayed until adulthood. As an example, the overall incidence of IM in the United States is approximately 1 per 50000 to 100000 persons per year, but in young adults, the incidence rises to about 1 per 100 per year (Plotkin, 1992). While most cases resolve spontaneously, life-threatening complications have been reported (Gold et al., 1995). Originally IM was called the kissing disease and had long been suspected to be caused by an orally transmitted agent (Hoagland, 1955). Following its link to EBV, Gerber et al (1972) detected EB viral particles in throat washings and saliva of IM patients and healthy individuals with a previous history of EBV infection. These EBV-infected patients were capable of transforming umbilical-cord lymphocytes into lymphoblasts with unlimited growth potential in culture (Gerber et al., 1972). Subsequent studies using in situ hybridization for viral DNA and for lytic RNA reported the presence of viral replication in desquamated epithelial cells present in the throat washings of IM patients and in epithelial cells lining the paratid duct (Sixbey et al., 1984). Although these studies suggest that the productive viral replication of EBV occur in the epithelial cells of EBV infected individuals, the primary target for the orally transmitted virus is not well defined. Identification of the EBV receptor on epithelial cells or cell lines has been most difficult. A 200-Kd glycoprotein expressed on the surface of epithelial cells was reported to be possibly the EBV receptor based on its

antigenic relatedness to the 145-Kd CD21 molecule on B cells (Young et al., 1989). However, most CD21-specific monoclonal antibodies did not recognize this 200-Kd glycoprotein (Young et al. 1986).

1.5.2 Persistent infection

Herpesviruses are characterized by their ability to persist for the life of the infected host (Klein and Klein, 1985). The molecular and cellular mechanisms underlying the persistence are only now beginning to be understood. Much thinking about EBV persistence has been fueled by observations on tumor cells and in vitro infected cell lines. Studies in vivo have been hampered by the very low frequencies of virus-infected B-cells and the fact that variable numbers of genome copies are present in infected cells depending on the form of infection. The proliferative potential of EBV-infected cells and life time exposure through persistence are believed to be the major predisposing factors in the development of EBV-associated malignancies.

The ability of EBV to infect mature resting B cells in vitro and induce their immortalization characterized by unlimited potential growth has been intensively studied; however, little is know about the nature of persistence in vivo (Klein, 1994). Several studies in the 1980s suggested that primary EBV infection take place in oropharyngeal cells and that this is the site of virus persistence and replication (Allday and Crawford, 1988). This hypothesis was based on the assumption that EBV persistence is similar to that of the papillomaviruses, which remain latent within the basal epithelial cell layer and replicate in a differentiation-dependent manner. In addition, the close proximity of B-lymphoid tissue to oropharyngeal epithelium would provide a route for spread of EBV from epithelium to B-cells (Young et al., 1991)

Several lines of evidence have recently challenged the earlier model of EBV persistence in epithelial cells. Niedobitek et al (1992) using Epstein-Barr virus-encoded RNA, (EBERs)-specific in situ hybridization showed that while EBV genomes and gene products are readily identified in the extrafollicular B-lymphoid blast, there is no evidence of epithelial cell infection by EBV in IM tonsils (Niedobitek et al., 1992).

Earlier studies showed that in bone marrow-transplant recipients whose resident laemopoietic tissue has been destroyed, eradication of EBV has occurred in some cases (Gratama et al., 1988). Moreover, treatment of IM or EBV-seropositive patients with an anti-viral agent (acyclovir) lead to a reduction of oropharyngeal virus shedding while the number of EBV-carrying circulating B cells remained constant (Yao et al., 1989). Results from these studies supported the idea that latently infected B cells are necessary and sufficient for persistence and that continued epithelial infection may depend upon seeding from the lymphoid reservoir.

The nature of EBV latency in the B lymphocyte pool of healthy virus carriers is not fully understood. Researchers have employed several types of assays to study the ability of EBV in establishing persistence in B cells. The only model for EBV latency in normal cells is the immortalization of B cells obtained by in vitro infection. Earlier studies concerning EBV latency where based on the use of B cell lines derived from peripheral blood (Yao et al., 1985). In this experimental model, EBV-induced outgrowth occurs predominantly by an indirect route involving release of virus and reinfection in culture (Rickinson et al., 1985). Therefore, only infected-cells with the capability to release viruses could be studied while EBV-infected cells that do not release infectious viruses would not be detected and studied.

On the other hand, some researchers used DNA polymerase chain reaction (PCR) to analyze the EBV persistence (Satio et al., 1989; Wagner et al., 1992). Indeed, PCR assays were only sensitive enough to measure the relative genome copy number. Whereas latently infected cells in vitro can contain multiple copies of the viral genome that can range from 5-500 (Sugden et al., 1979), the cells that contain the replicating virus have thousands of genomes (Summers and klein, 1976). Therefore, PCR assays could only compare relative burdens of viral DNA. As an example, It is known that in immunosuppressed individuals, the viral genome burden increases. Nevertheless, PCR-based assays can not distinguish whether this increase is due to an absolute increase in the number of latently infected cells or to an increase in the number of viral genomes per cell that result from viral replication. Therefore, PCR can not be used meaningfully to measure and analyze persistent infection.

A resent study by Khan et al (1996) reported that the absolute frequency of EBV-infected B cells in the peripheral blood of healthy carriers is almost stable and individual specific (Khan et al., 1996). In this report, the author shows that despite the presence of over 10-fold variation between individuals, a particular subject has a specific and stable frequency over the course of 1 to 3.5 years.

Findings that EBV was detected in certain T cell lymphomas (Jones et al., 1988) made the EBV persistence analysis much more complicated and raised the possibility of a greater tropism for the EBV in vivo than previously thought.

1.6 EBV-associated malignancies

The demonstration of herpesvirus-like particles in human lymphoblasts derived from a Burkitt's lymphoma in 1964 by Epstein and colleages marks the starting point of an active research in human tumor viruses. Since then, EBV has been subjected to intensive research analysis. EBV shows a remarkably strong association with at least five types of human malignancies and, as such, is a prominent example of a human tumor virus. Most tumors arise in long-term virus carriers many years after primary EBV infection, reflecting the multistep nature of the oncogenic process and its culmination in the malignant conversion of a single cell within the virus infected pool. Importantly, tumors such as BL, NPC, and posttransplant immunoblastic lymphomas are strongly associated with EBV infection and are consistently EBV genome-positive. Even though EBV is just one of several factors required for tumor development, the strong association with EBV implicates the virus as an etiological agent that is likewise playing an active role in the pathogenesis of these particular diseases.

1.6.1 Burkitt's lymphoma

Burkitt's lymphoma (BL), an aggressive B-cell tumor, is one of the most common childhood malignancies. BL has two characteristic features. First, an unusual high incidence ranging from 5 to 10 cases/ 10^5 per year over the first 15 years of life, and second, a marked geographic distribution that is determined by climatic factors and the incidence of malarial infection (Magrath, 1990).

There are two major subgroups of BL, endemic and sporadic, that differ in their geographic distribution. The high incidence or endemic BL is classically a childhood malignancy that is restricted to certain parts of equatorial Africa and costal New Guinea (Magrath, 1990). Importantly, all

tumors of endemic form display one of three reciprocal translocations between C-myc locus and either the immunoglobulin heavy chain locus (80% of the tumors) or one of the light chain loci (Bernheim et al., 1981).

There is a strong association between EBV infection and the endemic form of BL. In virtually every endemic tumor analyzed to date, all the malignant cells are EBV genome-positive with a monoclonal EBV episome (Neri et al., 1991). Early work of Henle et al showed that titers of anti-VCA and anti-EA antibodies in the serum of endemic BL pateints were considerably higher than in control groups (Henle et al., 1969; Henle and Henle, 1979). Moreover, seroepidemiological studies in endemic areas showed that individuals who subsequently developed BL had significantly raised anti-VCA titers months or years before the clinical onset of the tumor (Geser et al., 1982).

The other subgroup of BL is known as sporadic BL. Sporadic BL is now known to occur worldwide but with 50- to 100-fold lower incidence than that of the endemic disease. This form is best characterized in Europe and the USA where the tumors have a slightly higher age peak and a different pattern of presentation compared to endemic BL (O'Conor et al., 1965). In contrast to endemic BL, 15-25% of the sporadic BL tumors from Europeans and North Americans are EBV genome-positive (Magrath, 1990). Interestingly, as in the endemic disease, tumors of BL which are EBV-positive possessing a unique viral episome that is present in every tumor cell (Neri et al., 1991).

A third form of BL was recognized following the epidemic of the human immunodeficiency virus (HIV) infection over the last decade. It was noticed that in Western societies, HIV-infected individuals were susceptible to a range of B-cell malignancies with different pattern of presentation that tend to appear earlier in the course of progression towards AIDS before the

impairment of host immune function (Pedersen et al., 1991). Only a minority (30 to 40 %) of AIDS-BL are EBV positive (Ballerini et al 1993). AIDS-BL tumors display monoclonal EBV episomes (Neri et al., 1991), and are associated with both type 1 and type 2 EBV strains (Boyle et al., 1991).

1.6.2 Nasopharyngeal Carcinoma

Carcinomas of nasopharyngeal epithelium (NPC) are seen in all parts of the world but vary in incidence between different population groups. In contrast to Europe and North America, where NPC is a rare disease and histologically classified as a well-differentiated carcinoma. NPC is one of the most common cancers in Southern Asia, particularly among the Southern Chinese. In such areas almost all of the tumors are either poorly differentiated or undifferentiated NPC.

An association between EBV and NPC was first described by Old et al in 1966 (Old et al., 1966). This association was based on the observation that IgA antibodies directed against the EBV early antigens and VCA were elevated in pateints with NPC compared with those in appropraite controls (Zhu et al., 1986). Moreover, DNA-DNA reassociation and in situ hybridization studies indicated that the EBV genome could be uniformly detected in NPC tumor cells. Immunofluorescence staining first demonstrated the EBNA positivity of NPC tumor cells (Wolf et al., 1973). Early studies indicated the presence of EBNA1 in nearly all cases while, LMP 1 and LMP 2A could be detected in approximately 75% of EBV positive NPC tumor cells (Young et al., 1989).

1.6.3 Hodgkin's Disease

Hodgkin's Disease (HD) is seen worldwide but is particularly common in the western world, where it is the most frequently occurring malignant lymphoma. Several epidemiological aspects of HD, including its early age peak and predilection for high socioeconomic groups, are suggestive of a disease caused by delayed exposure to a rather common infectious agent (Gutensohn and Cole, 1980).

An association between EBV infection and HD was first suggested by serological studies. It was noticed that anti-VCA antibody titers were significantly raised in serum samples from patients with HD at least 3 years before the development of tumor, suggesting that deregulation of the EBV-host balance had preceded the appearance of the malignant clone (Mueller et al., 1989). However, the detection of EBV DNA, RNA, and protein in pathologic specimens from HD pateints were the key findings which irrevocably linked EBV to HD (Anagnostopoulos et al., 1989; Herbst et al., 1991). Studies by Munoz (1978) and others showed that individuals with a history of IM had a two- to four-fold increased risk of subsequently developing HD, the risk being greatest within the first 3 years of the viral illness (Munoz et al., 1978; Rosdahl et al., 1974). Overall, 40%-50% of HD tumors in the Western world are EBV-positive, and type 1 viral strains appear to be present in the great majority of these cases (Jarrett et al., 1991).

The latent-gene expression in EBV-associated HD is usually very similar to that seen in the majority of NPC cases. Therefore, all EBV positive Reed-Sternberg cells express the EBER RNAs (Wu et al., 1990), EBNA1 in the absence of other EBNAs (Grasser et al., 1994), and an unusually high level of LMP1 (Herbst et al., 1991).

1.6.4 T-cell lymphoma

The detection of EBV DNA in the circulating T cells of an infant with chronic IM-like symptoms (Kikuta et al., 1988), and in the occasional T-cell lymphomas (Jones et al., 1988) was the first indication that EBV, long considered a strictly B lymphotropic infectious agent, could under certain circumstances enter the T-cell compartment. Several studies have reported the presence of the EBV receptor, CD21, on particular mature (Fischer et al., 1991) or immature T-cell population (Watry et al., 1991)

Using sensitive in situ hybridization, at least three distinct T-cell tumors have been linked to the EBV infection. These include VAHS-associated T-cell lymphoma, Nasal T-cell lymphoma, and peripheral T-cell lymphomas of angioimmunoblastic-lymphadenopathy-like type (Pallesen et al., 1993; Su et al., 1991). The role of EBV infection in the pathogenesis of peripheral T-cell lymphomas remains unclear. Although infected by EBV to a significantly larger extent than described for B-cell non-Hodgkin's lymphoma, the partial infection of the tumor cell population and the relatively low LMP-1 expression pattern (Korbjuhn et al., 1993) suggests an uncertain role for the virus in tumorigenesis.

1.6.5 Immunoblastic lymphoma

The lymphoid neoplasm associated with EBV infection can occur in the setting of immunosuppression. An immunological defect affecting either the specific or non-specific cytotoxicity directed against EBV would lead to important pathogenic abnormalities thus allowing progression to a chronic or lethal B cell proliferation. This would be best illustrated by the association of the EBV infection to lymphoproliferative neoplastic diseases. B-cell lymphoproliferations are seen in three different groups of immunologically compromised patients.

In the mid-1970s, Purtilo and colleagues identified a family in which several young male children had succumbed to a fatal IM-like syndrome (Purtilo et al., 1975). This syndrome was later known as Purtilo's syndrome or X-linked lymphoproliferative syndrome (XLPS). Individuals with XLPS suffer from a genetic disorder with a selective immunodeficiency towards EBV. After acute EBV infection, approximately 75% of the males with XLPS develop a fatal IM within a few weeks, survivors then carry a very high risk of developing lymphomas (Purtilo, 1984). Immunologically, Okano et al (1984) reported that T cells from individuals with XLPS produce very low amounts of IFN-γ, which might be involved in the impairment of EBV-specific T-cell functions (Okano et al., 1989).

Second, immunoblastic lymphomas occur in recipients of solid organ transplants 10-1000 times more frequently than in the normal population (Hummel et al., 1995). Post-transplantation lymphoproliferative disorder (PTLD) is a well-recognized, frequently fatal complication of immunosuppression. The dramatic increase in the incidence of this type of immunoblastic lymphoma in recent years has been attributed to the wide usage of immunosuppressive agents such as cyclosporin A (Crawford et al., 1980). Since most cases of post-transplant lymphoma appear to be EBV genome-positive, EBV is believed to have an important role in the pathogenesis of PTLD (Purtilo and Klein, 1981; Knowles et al., 1995). The development of PTLD in immunosuppressed individuals is believed to result from inadequate T-cell control over EBV-driven B-cell proliferation (Randhawa et al. 1992). Importantly, the proliferating cells of lymphoma lesions usually express the full spectrum of EBV latent proteins and cell surface markers similar to those seen on in vitro transformed LCLs (Gratama et al., 1991).

Individuals infected with the HIV constitute the other group with an increased risk of developing immunoblastic lymphoma (Ross et al., 1985).

These patients show the same spectrum of changes in the EBV-host balance as seen in genetically immunosuppressed patients such as individuals with XLPS. A substantial proportion of HIV carriers develop oral hairy leukoplakia (OHL), a wartlike lesion on the lateral borders of the tongue which proved to be an epithelial focus of EBV replication (Greenspan et al., 1985). Importantly, despite the fact that 70% to 80% of the immunoblastic lymphomas that occur in AIDS patients are EBV-linked (Boyle et al., 1991), the nature of viral gene expression has still to be fully resolved. Interestingly, Neri et al (1991) found while working on EBV-positive AIDS-related lymphomas, that all had clonal EBV infection (Neri et al., 1991), indicating that EBV infection occurred before clonal B-cell expansion.

1.6.6 Other EBV-related malignancies

Using EBER in situ hybridization technique, researchers were able to link EBV to several other malignancies. These tumors have been described in the stomach (Shibata et al, 1994), salivary glands (Chan et al., 1994), lung (Butler et al., 1989), and thymus (Wu and Kuo, 1993). The elucidation of the precise role of EBV in the development and maintenance of human neoplasia is still a challenge to be solved by future research.

CHAPTER 2

Immunological responses to EBV infection

Patients with acute IM have been seen to have a high number of EBV-infected B cells in their peripheral blood that are capable of continuous growth in vitro. Additionally, after primary infection, normal EBV-seropositive individuals have a small but detectable number of circulating B cells latently infected with EBV that have the capability to grow indefinitely when cultured in vitro (Rocchi et al., 1977). Yet these individuals very rarely develop neoplasms. This has been attributed to the vigorous host immune response that develops during the course of the disease. In this chapter, I will describe the non-specific and specific humoral and cellular mediated immune responses directed against EBV infection.

Researchers have described several important mechanisms that are able to kill EBV infected cells or suppress their clonogenic potential. These mechanisms include mucus, interferon, natural killer (NK) cells, neutralizing antibodies, antibody-dependant cell-mediated cytotoxicity, and human leukocyte antigen-restricted or –nonrestricted EBV-specific cytotoxic T cells (Okano et al., 1988).

2.1 Non-specific immune response

The first line of non-specific defense against EBV was found to be in the oral cavity, where EBV transmission occurs. Aya et al (1980) investigated the presence of unknown protection mechanisms against EBV infection in the

oral cavity. The author showed that a substance of low molecular weight present in saliva with the ability to inhibit the growth of EBV-converted B-lymphocytes through its cytotoxic action (Aya et al., 1980). This suggested that saliva, secreted from the oral cavity, is the first barrier against EBV invasion.

2.1.1 Interleukine-mediated

After EBV infection of B-lymphocytes, the interferon is liberated by the virally infected B-cells, activated T and natural killer (NK) cells (Thorley-Lawson, 1981). Interferon may control the EBV-induced B-cell immortalization by antiviral replication activity and boosting of NK cell activity (Portilo et al., 1985; Zarling et al., 1992). Indeed, interferon and NK cells may act synergistically to protect against EBV. Andersson et al (1985) have shown that purified interferon can activate NK cells that will gain the ability to eliminate EBV infected cells in vitro (Andersson et al., 1985).

Monocytes constitute an important element of the immune system that is known to play a major role in the primary intracellular infection mainly through cytokine secretion (Gosselin et al., 1992). IL-6, IL-1, IL-12 and TNF-α which are mainly produced by monocytic cells (Navarro et al., 1989), can act on a wide range of cell types and induce production of other cytokines (Hirano et al., 1990). Several laboratories have shown that monocyte-derived IL-12 is required, yet insufficient for optimal production of IFN-γ by native NK cells. However, the combination of IL-12 and the T-cell-derived IL-2 cytokine was seen to provide a strong stimulus for IFN-γ production by NK cells (Tripp et al., 1993). Monocytes have two independent mechanisms for destroying tumor targets. First, in the presence of Fc receptors, monocytes participate in the ADCC-mediated destruction of tumor cells. Second, like NK cells, monocytes are capable of destroying tumor targets by a mechanism

known as spontaneous cytotoxicity which can be upregulated by a variety of stimuli such as IFN-γ and IL-2.

2.1.2 Cellular-mediated

NK cells are large granular lymphocytes (LGL) that lack rearrangement of both T-cell receptor and immunoglobulin genes and constitutively express a number of cell surface cytokine receptors. They constitute 10-19 % of the mononuclear cell population in peripheral blood (Hannet et al., 1992) with the capability of spontaneous cytotoxicity (Robertson and Ritz, 1990). Earlier studies (Blazar et al. 1980; Patel and Menezes, 1982) suggested the importance of NK cells in the immune regulation against EBV infection. These researchers showed that cells infected with EBV in vitro became more sensitive to NK lysis than uninfected cells (Blazar et al., 1980; Patel and Menezes, 1982). Moreover, Patel et al (1982) suggest that NK cells may play an important role in controlling EBV infection in patients with IM in the acute phase of the disease and authors gave evidence that the antiviral immune response directed against EBV-infected cells during the acute phase of IM was not specific to EBV infected cells. They showed that whole blood lymphocytes from IM patients were efficiently able to kill EBV-genome positive cell lines (Raji, B35M, and P3HR1), whereas, removal of Fc-bearing cells from whole lymphocytes significantly diminished this cytotoxic activity (Patel et al., 1982). In addition, significant cytotoxic activity was found in the whole blood lymphocytes compared to enriched T-lymphocytes from IM patients when the cytotoxic potentialities were tested towards EBV-genome-negative (BJAB) and EBV negative (K562) target cell lines (Patel et al., 1982). The importance of NK activity is further documented in the study done by Biron et al (1989) in which he reported the case of a patient with a total

absence of NK cells suffering consequently from life-threatening herpes virus infections (Biron et al., 1989).

Antibody-dependent cellular cytotoxicity (ADCC). is thought to play a major role in controlling viral infections and in immunity to tumors induced by oncogenic viruses (Kohl and Loo, 1982). The protective nature of ADCC has been documented in a number of human studies (Imir et al., 1976; Khyatti et al., 1994). This immune mechanism involves the interaction of antibodies with the low affinity receptor (FcRIII, CD16) located mostly on NK cells, monocytes, and antigenic structures on the surface of target cells (Lanier et al., 1983). Therefore, binding of NK cells to targets via an antibody bridge result in the rapid cytolysis of target cells. In terms of EBV infection, it was found that ADCC had the ability to control the spread of the virus in an infected individual. Prospective studies of patients suffering from either Burkkit's lymphoma or nasopharyngeal carcinoma have reveald that patients with high titers of anti-EBV ADCC activity had a better long term prognosis (Neel et al., 1984; Pearson et al 1979).

Although there is a strong antibody response to EBV, T cells play an essential role in controlling both the primary and the persistent phases of EBV infection and preventing the development of immunoblastic B-cell lymphomas (Strang and Rickinson, 1987; Musucci and Ernberg, 1994). The significant increase in the lymphocyte count during the acute phase of EBV infection provoked an early suspicion that a cellular response could be important in controlling EBV infection. The observation by Sheldon et al (1973) that these lymphocytes were not EBV-infected B cells but lymphoblasts of thymic origin reinforced the immunological role of these atypical lymphocytes (Sheldon et al., 1973). Earlier studies indicated that the expanded cells were CD8+ T cells, with 70 to 75% expressing the activation marker, HLA-DR (Tomkinson et al., 1987). Incubation of blood lymphocytes with EBV-infected autologous B

cells, in vitro, was seen to show a similar lymphoblastic reaction (Viallat et al., 1978).

The preferential lysis of EBV-infected targets led to the concept of a novel antigen expressed on B cells after EBV infection rendering them sensitive to recognition and lysis by immune T cells. A study by Svedmyr and Jondal (1975) suggested that lymphocytes that develop during the acute phase of IM have a specific cytotoxic function against EBV-genome-positive B-cells through the lymphocyte-detected membrane antigen (LYDMA) (Svedmyr and Jondal, 1975). However, several studies provided evidences that challenged the concept of EBV specificity for the acute IM alloreactive cytotoxic response (Klein et al., 1981; Patel et al., 1982; Rickinson et al., 1985; Sullivan, 1987).

Several groups have observed the emergence of suppressor T cells capable of inhibiting imunoglobulin production by mitogen-stimulated B cells during IM (Hyines et al., 1979; Tosato et al., 1979). Wang et al (1987) showed that the suppression defense mechanism is an independent mechanism of immune defense to EBV infection during IM (Wang et al., 1987). They succeeded to isolate a clonal population of T cell origin from the peripheral blood of IM patients that could markedly inhibit EBV activated B cells without detectable cytotoxic activity. However, similar clones could not be isolated from the blood of any normal-seropositive individual examined. Interestingly, by measuring cytokines in the supernatants of the cloned suppressor cells, they found that low levels of gamma interferon were present in all suppressor T-cell clones examined. The authors suggested that gamma interferon secretion by suppressor T cells from IM patients peripheral blood might be the cause of inhibition, because recombinant gamma interferon has been shown to profoundly suppress EBV-induced Ig production in vitro (Lotz et al., 1985).

2.2 Specific immune response

2.2.1 Humoral-mediated immune response

The presence of EBV in the epithelial cells and B cells promotes the activation of a strong humoral response consisting of antibodies to a large variety of virally encoded antigens. During the development of anti-EBV-related immunity in acute mononucleosis, the humoral response is directed primarily towards viral antigens of the lytic cycle including: the membrane antigen complex (MA), the early antigen (EA, most frequently to the diffuse component), and the viral capsid antigen (VCA) (Henle et al., 1970). The neutralizing antibodies directed against components of the MA complex are probably the most important humoral response. This is due to the fact that anti-MA antibodies have the capacity to prevent generalized viraemic spread of the infection.

An earlier study, Henle et al (1979) reported that the anti-MA secreted in the acute phase of EBV infection was directed towards gp85 rather than gp350/220 (Henle and Henle, 1979). However, the antibody response to the latter component was shown to be more potent (Thorley-Lawson and Geilinger, 1980). Therefore, the lack of strong anti gp350/220 reactivity probably explains the inefficiency of the humoral response during primary EBV infection. Acute IM is also characterized by a pronounced IgM antibody response to autoantigens and heterophile antigens presumably associated with the well-documented role of the virus as a polyclonal B-cell activator.

Another interesting feature of IM serology is the pattern of anti-EBNA reactivity. Even though IgM anti EBNA1 antibodies are easily detectable in IM sera by immunoblotting, the IgG response to EBNA2 appears before the appearance of IgG anti-EBNA1 as detected by anti complement immunofluorescence (Henle et al., 1987; Rhode et al., 1987). This was explained originally by Henle et al (1974). The study reported that this early response towards EBNA2 might reflect the delayed destruction of latently infected cells in vivo (Henle et al., 1974). Another explanation based on the poor response of CD8+ T cells towards EBNA indicates that EBNA1 epitopes may not be available for the immune system during IM. Importantly, after primary infection, although they persist for life, the anti EBNA and anti VCA responses appear to have little if any protective role (Moss et al., 1992).

As IM patients recover from clinical symptoms, the IgM response is significantly reduced while the IgG response in serum remains at a low level and is maintained throughout the persistent infection (Henle and Henle, 1979). The presence of a high level of viral replication during acute infection in the oropharynx explains the strong antibody response seen in this phase, nevertheless, the importance of the humoral immune response in terms of viral control in the primary infection remains uncertain.

It is worth noting that a transient IgA response to the gp 350/220 antigen (Sixbey and Yao, 1992), may actually have a role in controlling of the virus spread to epithelial cells (Gan et al., 1994).

2.2.2 Cellular-mediated immune response

CD8+ HLA class l-restricted CTL to immediate early or early lytic cycle antigens are frequently detectable in the blood of IM patients. Steven

and coworkers illustrated in a recent article the presence of a cell-mediated response directed against an epitope in the immediate early protein BZLF1 (Steven et al., 1996). A class II-restricted, CD4+ CTL response may also play an important role in controlling primary infection. Early in vitro experiments showed that cocultivating unfractionated mononuclear cells with the irradiated autologous LCLs from EBV-seronegative individuals resulted in generation of a primary EBV-specific, CD4+ CTL response (Misko et al., 1991). Additionally, by identification and isolation of a number of CD4+ T-cells directed towards gp350 epitopes (Wallace et al., 1991), it has been suggested that this CD4 T-cell subset may play an essential role in the cellular immune response against EBV infection through lymphokine secretion (Pearson et al., 1987). A more recent study reported by White (1996) described the presence of CD4+ HLA class II-restricted CTL activity mediated by a rare CD4+CD8+ T-cell subset that recognized an early lytic cycle antigen, the BHRF1 protein (White et al., 1996).

As the clinical manifestation of IM resolves, EBV-specific HLA class I-restricted CD8+ cytotoxic T cells emerge as the dominant population of EBV-reactive T cells (Moss et al., 1987). As is the case with other antiviral cell-mediated responses, the predominant CD8+ effectors recognize small peptide fragments of endogenously synthesized viral proteins presented on the cell surface in the peptide-binding groove of HLA class-I molecules. The existence of a cell-mediated immune control over the largely non-productive EBV infection in B cells has been recognized for several years. However, in the absence of animal models of persistent EBV infection, the relative importance of the cell-mediated response has not been evaluated experimentally in vivo. Nevertheless, evidence from in vitro studies suggests an essential role for the virus-specific CD8+ CTLs. These observations revealed the existence of EBV-specific memory T cells through their capacity to regulate the course of virus-induced transformation of B cells (Moss et al.,

1987; Rickinson et al 1979). This CTL activity was only observed in lymphocyte culture from EBV-seropositive individuals and was specific for MHC-mediated EBV-infected cells since autologous mitogen-activated blasts were not lysed (Misko et al., 1990; Misko et al., 1991). Importantly, even when the initial expansion of latently infected growth transformed B-cells is brought under control, the virus is never eliminated from the infected B-cell pool. Reversion of some growth-transformed B cells back to the resting state (Miyashita et al., 1995), and down regulation of many of the virus latent antigens that constitute the major targets for CTL attack is thought to be the key event in the establishment of virus persistence.

As was mentioned previously, in the mid 1970s Svedmyr et al (1975) suggested that the EBV-specific CTL response is directed to an EBV-associated LYDMA, whose existence was first postulated to explain the appearance of virus-specific CTL function in the blood of IM patients (Svedmyr and Jondal, 1975). Subsequently, the attention of scientists was shifted towards latent the protein, EBNA, as a source of CTL epitopes. This change was due to two important findings: First, the growing information in the field of immunology concerning peptide processing and presentation in association with the HLA class I molecule. Second, the influenza virus model in which CTLs are directed towards nuclear antigen (Bastin et al., 1987).

Early results showed indeed that EBNA proteins could act as a target for CTL-cell-mediated immune response (Szigeti et al., 1989). Although EBV is a complex virus with the capacity to encode 80-100 proteins, the dominant targets for CLT response have been mapped to EBV latent membranes. To date more than 19 CTL epitopes have been described at the peptide level and are located in EBNA1 through EBNA4, EBNA6, and LMP2 (Brooks et al., 1993; Burrows et al., 1994; Burrows et al., 1990; Lee et al., 1993; Schmidt et al., 1991). It is important to note that the nature of antigen processing and

presentation by class-I molecules means that all 9 EBV latent antigens constitutively expressed in LCL cells are potential sources of immunogenic peptide epitopes, irrespective of the native location of these proteins within the infected cell. In addition, since antigen presentation selects peptides with high affinity for a particular peptide binding groove, the identity of the dominant peptide epitopes and hence of the viral proteins from which they are derived, differs between individuals and is dependent upon each individual's HLA class-I antigen type (Gaviolo et al., 1993).

New advanced techniques have provided fundamental insight into the fine detail of the immune system. Recently, two technical advances in the immunology field have facilitated these studies. First, the construction of recombinant vaccinia viruses capable of expressing individual EBV latent antigens (Murray et al., 1990). The second technique was the establishment of an EBV-negative host cell, which was efficiently infectable with vaccinia virus (Khanna et al., 1991). Using these two important tools, scientists were able to construct individual EBV/vaccinia recombinants for each of the nine immunogenic virus latent genes. Based on studies done on a large panel of virus immune donors with a wide range of HLA types, scientists were able to identify and localize several target antigens for CTL response that controlled EBV infection (Murray et al., 1992; Khanna et al 1992). These works lead to four important conclusions. First, a substantial part of the EBV-specific CTL response was directed against epitopes from the EBNA3A, 3B, and 3C family of latent proteins. Second, more than 30 different CTL specificities restricted through eight different classes I alleles were defined. Interestingly, HLA-A2, B7, B8, B40, and B51 alleles were found to present more than one distinct CTL epitope derived from different latent antigens. Third, surprisingly, using the same molecular technique, the specificity of a large number of EBV-specific CTL-clones (68%) could not be defined. Two possible explanations were given for the last observation. a) the undefined epitopes are located

within EBV latent antigens other than those encoded and tested by the vaccinia constructs. b) the antigen associated with the EBV replication cycle could also include epitopes with the ability to activate the CTL immune response. Fourth, the extreme rarity of CTL response towards EBNA1 antigen epitopes.

The basis for the rarity of cellular responses to EBNA1 was further analyzed recently. Levitskaya et al (1995) suggested that the presence of a 200 residue long internal glycine-alanine repeat domain encoded by EBNA1 prevented processing and presentation of the EBNA1 epitopes through the HLA class I pathway (Levitskaya et al., 1995). Internal glycine-alanine repeats were conserved in all normal EBV isolates, nevertheless, they were not required for maintenance of the episomal viral genome in the latently infected cells (Yates and Camiolo, 1988). Moreover, it was shown that insertion of the glycine-alanine domain into the EBNA 3B sequence significantly reduced the efficiency with which epitopes from this latent protein were presented for CTL recognition (Levitskaya et al., 1995). Surprisingly, rare CD8+ HLA class II-restricted CTLs against EBNA1 epitope have been detected in the memory of sero-positive individuals, suggesting that the protection mechanism for processing and presentation provided by internal repeats may on occasion be overridden (Rickinson and Moss, 1997). On the other hand, Khanna et al (1995) detected CD4+ HLA class II-restricted CTLs directed against EBNA1 epitope which recognize LCL cells to which EBNA1 has been supplied as an exogenous antigen (Khanna et al., 1995).

Another unusual feature of antigen processing and presentation in the EBV infection system is the rarity of response to EBNA2 and LMP1 that remain unexplained despite the fact that each of these proteins contains peptide epitopes capable of binding to a variety of HLA alleles (Stuber et al., 1995).

CHAPTER 3

Interlukin (IL)-15

3.1 Discovery and characterization of IL-15

The first evidence that pointed to the possible existence of a non-IL-2 T-cell growth factor was presented by Thomas A.Waldmann in two companion articles (Burton et al., 1994; Bamford et al., 1994). The first article described the preliminary purification and characterization of an IL-molecule called IL-T which was found to support the growth of the murine IL-2-dependent CTLL-2 cell line as well as the human cytokine-dependent T-cell line kit-225. The second article provided further explanation for the high expression of IL-2R that was found on leukemic cells during the late-phase of ATL (Bamford et al., 1994). This cytokine provisionally designated IL-T was isolated amidst an effort to determine the pathogenic mechanism underlying the IL-2 independent proliferation of leukemic cells during late-phase ATL (adult T-cell leukemia) caused by HTLV-1 (Burton et al., 1994). HuT-102 (an ATL cell line) supernatant was capable of supporting the growth of the cytokine-dependent murine T cell line CTLL-2 and Mik-ß1, a monoclonal antibody that binds specifically to the IL-2Rß subunit, was found to abrogate the proliferation of kit-225 in the presence of HuT-102 culture supernatant (Bamford et al., 1994).

A couple of years later while measurement of cytokine activity in the supernatant of a simian kidney epithelial cell line CV-1/EBNA, Grabstein group isolated and purified a soluble factor with an ability to support the

growth of the IL-2 dependent cell line CTLL (Grabstein et al., 1994). The isolated protein formed a band of 14-18 Kda on SDS-PAGE and was named IL-15.

IL-15 shared many characteristics with IL-T, including a molecular mass of 14-15 kDa as well as the utilization in T and NK cells of the β and γ_c subunits of the IL-2R. The use of anti-cytokine antibodies gave evidence that IL-T was identical to IL-15 (Bamford et al., 1996).

The human IL-15 gene was mapped to chromosome 4 band 4q31. IL-15 was found to be highly conserved, it has 95% aa sequence homology with simian IL-15 and 73% with the murine IL-15 (Anderson et al., 1995). Several other human growth factors and cytokine genes map to the same arm of h-chromosome 4, in particular, the IL-2 gene that is mapped to 4q26-4q27. Interestingly, although IL-15 and IL-2 was found to be structurally similar, and are both members of the helical cytokine family, they have no sequence homology (Grabstein et al., 1994).

3.2 Cellular expression of IL-15

Several reports indicate that monocytes are the main cell type involved in IL-15 production during infection (Seder et al., 1995; Ferrick et al., 1995; Doherty et al., 1996; Kacani et al., 1997). Other cellular sources for IL-15 were also documented such as epidermal Langerhans cells, human keratinocytes, blood dendritic cells, Dermal fibroblasts, umbilical vein endothelial cells. All these cells constitutively expressed IL-15 mRNA (Jonuleit et al, 1997; Blauvelt et al., 1996; Mohamadzadeh et al., 1995; Mrozek et al., 1996). Furthermore, IL-15 mRNA was also detected in several

human tissues such as heart, lung, liver, kidney, placenta and skeletal muscle. However, normal activated T lymphocytes, the only currently known source of IL-2, lack the IL-15 mRNA (Grabstein et al., 1994).

3.3 Receptor Complex of IL-15

Both IL-15 and IL-2 share the ability to support the growth of various T cell lines, Ag dependent T cell clones (Tho, Th1, Th2) and activated normal T cells (Grabstein et al., 1994). Moreover, they both have costimulatory activity for proliferation and Ig production of human tonsilar B cells, and IL-15 can replace IL-2 as costimulator in an in-vitro antigen-specific antibody response, but IL-15 has no effect on resting B cells (Armitage et al., 1995).

IL-15 is also an efficient activator of NK cytotoxic activity (Giri et al., 1995), this ability to induce NK cytotoxicity is not mediated by IL-2, since neutralizing antibodies to IL-2 did not inhibit the activation of NK cells by IL-15. Also, both IL-2 and IL-15 combine with IL-12 for costimulation of production of interferon-γ and tumor necrosis factor-α by NK cells in a comparable level; however, the combination of IL-15 with IL-2 does not appear to have an additive or synergistic effect (Carson et al., 1994). It follows that, by sharing several biologic activities, IL-15 and IL-2 also share components of the IL-2R like other cytokines that belong to the four helix bundle cytokine family (Cosman 1993; Kondo et al., 1993).

The receptor for IL-2 is composed of at least three distinct subunits, the α chain (IL-2Rα), the β chain (IL-2Rβ), and the γ chain (IL-2Rγ). The genes encoding the three receptor subunits have been cloned, and their complete primary structures have been deduced (Taniguchi et al., 1983;

Nikaido et al., 1984). The human IL-2 Receptor α-chain, originally described as the Tac antigen, was identified as a 55-Kd membrane glycoprotein (p55) capable of binding IL-2 (Leonard et al., 1993) with low affinity (Hatakeyama et al., 1985). The IL-2 Receptor β chain (p75) is the subunit critical for receptor-mediated signaling. IL-2Rβ is linked to at least two intracellular signaling pathways that mediate nuclear proto-oncogene induction (Minami et al., 1993). The IL-2R γ (p64) is required for the receptor-mediated internalization of IL-2 (Takashita et al., 1992). Both IL-2Rβ and IL-2Rγ are type I membrane glycoproteins (Bazan 1990).

IL-15 utilizes both the β and γ chains of the IL-2 receptor for binding and signal transduction. Antibodies specific for either the β or the γ chain of the IL-2R block IL-15 induced responses in vitro, whereas antibodies specific for the α chain of the IL-2R have no inhibitory effect (Grabstein et al., 1994; Bamford et al., 1994; Giri et al., 1994). These and other studies are clearly demonstrate that IL-15 can utilize both the β and the γ chains, but not the α chain of the IL-2R, and that the β and γ chains are essential for signaling and efficient internalization of IL-15 in cells of hematopoietic origin (Grabstein et al., 1994; Giri et al., 1994; Giri et al., 1995; Kumaki et al., 1995).

IL-15 sharing of two receptor subunits with IL-2 does not reflect completely similar biologic activities. There are functional differences between the two cytokines (Grabstein et al., 1994; Giri et al., 1994; Carson et al., 1994) that may result from distinct interactions of IL-2 and IL-15 with IL-2Rβ and IL-2Rγ or the usage of different α chain. Several experimental evidence have shown that IL-15 and IL-2 might not bind with the same specificity or affinity to IL-2R e.g the proliferation of PHA-stimulated PBL in response to IL-2 is not affected by TU11, a mAb directed against the IL-2Rβ chain that does not interfere with IL-2 binding (Suzuki et al., 1993). However, TU11 does partially inhibit IL-15 stimulation of these cells, indicating that IL-

2 and IL-15 might bind to IL-2Rβ at slightly different epitopes (Grabstein et al., 1994). Another example Mikβ1, a mAb that blocks IL-2 binding to IL-2Rβ , also inhibits IL-2 stimulated proliferation of PHA blasts, but only blocks 75% of IL-15 stimulated proliferation (Grabstein et al., 1994; Jill et al., 1996).

In an effort to characterize the receptors for IL-15 and IL-2, scattered analysis of binding to CTLL cells and PHA-activated peripheral blood T cells revealed that IL-15 had only a single class of high-affinity binding sites whereas IL-2 had a high affinity and a large number of low-affinity binding sites (Grabstein et al., 1994; Giri et al., 1994). Experimental evidence pointed to the presence of another receptor subunit involved in IL-15 binding and signaling. IL-15 was found to bind with high affinity to B cell lines established from patients with X-linked severe combined immunodeficiency (Kumaki et al., 1995). Kumaki et al. suggested the probable presence of IL-15Rα since these cells did not express the IL-2Rγ chain and IL-15 was incapable of binding to IL-2Rβ alone (Giri et al., 1994).

IL-15Rα in the murine system was first isolated by Giri, JG. et al. and its cDNA characterized and molecularly cloned (Giri et al., 1995). It encodes a 58- to 60-KDa type I membrane protein with striking structural similarity to the IL-2Rα chain. Whereas IL-2Rα was found to be expressed only in the lymphoid cell lines, the murine IL-15Rα is expressed in a large variety of cell types, including B and T cell lines, a macrophage cell line, and thymic bone marrow stromal cell lines. The distribution of IL-15 and IL-15Rα mRNA suggests that IL-15 may have biological activities distinct from IL-2 (Giri et al., 1995).

As for the human IL-15Rα chain; three differentially spliced human IL-15Rα variants were isolated (Anderson et al., 1995). Similar to the murine IL-15Rα chain, the human IL-15Rα chains are capable of high affinity

binding of IL-15. Also, no role in signaling was observed in the cytoplasmic domain of hIL-15Rα, like that of IL-2Rα. At high concentrations, IL-15, like IL-2, is able to signal through a complex of IL-2Rβ and -γ in the absence of the α subunit. Importantly, like with the murine IL-15Rα, the distribution of expression of the human IL-15Rα is much wider than that of the IL-2Rα, suggesting a broader range of cellular targets for IL-15 (Anderson et al., 1995).

IL-15 appears to interact with IL-2 to allow cells to respond primarily to IL-2. This is shown by the rapid down-regulation of IL-15's own high-affinity binding sites (IL-15Rα) upon stimulation of human T and B cells. This down-regulation of high-affinity Il-15 binding sites is directly linked to a decrease in IL-15 responsiveness in T cells as measured by a reduced responsiveness to a physiological concentration of IL-15 as evaluated by tyrosine phosphorylation of JAK3 kinase; whereas IL-2Rα expression is upregulated by both IL-15 and IL-2 (Kumaki et al., 1996).

3.4 Regulation of IL-15 expression

IL-15 has a differential regulation for its expression compared to cytokines belonging to the same family such as IL-2 which is regulated at the level of message transcription and stabilization. The presence of upstream AUGs in the IL-15 5' UTR region was shown to control the expression of the protein. Analysis of the IL-15 message from the HuT-102 T cell line, showed that it consisted of a chimeric mRNA joining a segment of the R region of the long terminal repeat of HTLV-1 and the 5' untranslated region (UTR) of IL-15. The greater amount (6- to 10-fold) of protein expressed in HuT-102 cells as compared to activated monocytes, correlated with the lack in HuT-102 cells

of over 200 nucleotides of the IL-15 5' UTR including 8 of 10 upstream AUGs that are present in the normal IL-15 message. Thus, it was concluded that IL-15's transcription and translation was increased in the ATL cell line due to the presence of an HTLV-1-R fusion message that lacked many upstream AUGs (Bamford et al., 1996). In fact, it has been difficult to demonstrate IL-15 in the supernatants of the majority of the cells that express messages for this cytokine, despite its widespread expression in several human tissues (Bamford et al., 1996).

Further evidence confirming the presence of a negative regulatory role for IL-15 signal peptides in secretion of the protein was presented in a study by Onu, et al. (Onu et al., 1997) in which assessment of the control of IL-15 secretion was done by constructing an IL-15/IgG1 chimera which shows that by adding a foreign leader peptide, translation and secretion are increased. Hence, IL-15's synthesis and secretion appears to be controlled at multiple levels (translation and entry into secretory pathway), in addition to transcription (Bamford et al., 1996).

3.5 Pleiotropic function of IL-15

IL-15, like IL-2, is a chemoattractant and growth factor for T cells. Human blood CD4+ and CD8+ T lymphocytes migrate and are attracted by IL-15; although IL-15 does not appear to have any chemokinetic or chemotactic activity on human monocytes, neutrophils, or B cells (Wilkinson and Liew 1995; McInnes et al., 1996). Also, IL-15 transcription correlated with T cell expansion that accompanies human renal allograft rejection as demonstrated in renal biopsies (Pavlakis et al., 1996). When used at optimal concentrations, IL-15 rapidly induced memory (CD45RO+) CD4+ and CD8+

T cells and naive (CD45RO-) CD8+ T cells to express the CD69 activation marker followed by proliferation (Hirokazu et al., 1996).

IL-15 is an efficient activator of NK cytotoxic activity (Judith et al., 1995). In addition, the ability of NK cells to mediate antibody-dependent cellular cytotoxicity (ADCC) is augmented by IL-15 (Carson et al., 1994). Lymphokine-activated killer cell function (LAK) is generated following treatment with exogenous IL-15 of cultures of human PBMCs, large granular lymphocytes (LGLs), or purified $CD56^{dim}$ NK cells (Burton et al., 1994; Grabstein et al., 1994; Carson et al., 1994; Flamand et al., 1996; Gamero et al., 1995). IL-15 was found to activate human NK cells using components of the IL-2R and to synergize with IL-12 to significantly potentiate NK cell production of IFN-γ (Carson et al., 1994). Moreover, infection of PBMC with HHV-6 or HHV-7 enhanced in-vitro NK cell cytotoxicity via IL-15 induction (Flamand et al., 1996, Atedzoe et al., 1997). Furthermore, in PBMCs from HIV-infected patients at various stages of disease, neutralization of endogenous IL-15 resulted in diminished IFN-γ production in vitro (Seder et al., 1995). IL-15 has been also documented to play a role in the differentiation of NK cells. Culture of the bipotential T/natural killer IL-2Rβ+ TCR- progenitor cells of the murine fetal thymus in the presence of IL-15, resulted in the generation of functional NK cells (Leclerqe et al., 1996).

IL-15 costimulates the proliferation of PMA- or anti-IgM-activated, purified human B cells, but has no stimulatory effect on resting human B cells (Armitage et al., 1995).

3.6 Role of IL-15 in infection

The data generated from a number of studies suggests strongly that IL-15 have a role in the early immune response to infection. Monocytes, the main site of IL-15 synthesis, respond to intracellular infection. When activated by LPS, human monocytes induced interferon- (IFN-γ) production in NK cells in-vitro with concomitant detection of IL-15 and IL-12. Experimental evidence suggests that IL-15 synergizes with IL-12 and this is seen in the significant potentiation of NK cell production of IFN-γ in-vitro by a combination of IL-15 and IL-12 (Carson et al., 1994), also expression of IL-15 and IL-12 transcripts by monocytes following activation by LPS occurs within the same time frame (D`Andrea et al., 1993). Monocytes are the main cell type involved in IL-15 production during infection (Nishimura et al., 1996; Kacani et al., 1997; Jullien et al., 1997). Peripheral blood monocytes secreted IL-15 in response to infection with Mycobacterium leprae; in addition, resistance in tuberculoid patients was associated with a high expression of IL-15 mRNA and a Th1 cytokine pattern of cell-mediated immunity, as compared to susceptible lepromatous patients (Jullien et al., 1997). In HIV-1 patients IL-15 serum levels were found to be increased and correlated with the observed hypergammaglobulinaemia, studies in vitro showed that PBMC and monocytes produce IL-15 in response to HIV-1 virus (Kacani et al., 1997).

Apart from the ability of IL-15 to activate NK cells as an immune response to infection of monocytes with obligate intracellular pathogens and gram negative organisms (Carson et al., 1994; Carson et al., 1995; Tripp et al., 1993), IL-15 can augment the cellular immune response to infection through CD8+ and gamma-delta T cells. IL-15 was shown to prime naive CD8+ T cells that in the presence of soluble T.gondii Ag are activated and able to generate an Ag-specific proliferative response, thus conferring protection to mice against a lethal challenge with T.gondii (Imtiaz et al., 1996). The role of IL-15 in microbial infection has been associated in some murine models with an expansion in the gamma-delta T cell pool (Nishimura et al., 1996;

Leclercqe et al., 1996). Primary infection in mice with Salmonella cholerasuis leads to a γδ T cell proliferative response with expression of the β and γ chains of the IL-2R complex; when γδ T cells were stimulated in-vitro with rIL-15, they proliferated and produced IFN-γ and IL-4, no protein was detected (Nishimura et al., 1996). This points to a role for IL-15 in the early activation of γδ T cells during Salmonellosis. In humans, a report by Elloso et al., showed that in malarial infection, the proliferation of the γδ T cell subset which is normally dependent on CD4+ αβ T cells was induced by addition of exogenous IL-2, IL-4 or IL-15 to cultures of PBMC depleted of CD4+ αβ T cells (Elloso et al., 1996).

Taken together, the data points to an important role for IL-15 in protective immunity to bacterial, parasitic, and viral infections. Evidence for IL-15's implication in the viral immune response has only begun to be elucidated.

II. ARTICLE PRESENTATION

CHAPTER 4

IL-15 INDUCES EXPANSION OF CD3+CD56+ T CELLS AND INHIBITS THE OUTGROWTH OF THE EPSTEIN-BARR VIRUS-INFECTED/IMMORTALIZED PERIPHERAL BLOOD LYMPHOCYTES IN VITRO

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ABSTRACT

IL-15 is a recently discovered cytokine with T cell growth factor activity and capacity to enhance the cytocidal function of lymphocytes. In this study, we investigated the effect of IL-15 on immune control of the outgrowth of Epstein-Barr virus (EBV)-infected/transformed lymphocytes *in vitro*. Peripheral blood mononuclear cells (PBMC) from healthy donors were infected with EBV in the presence or absence of exogenous recombinant human IL-15, then cultured and examined for several weeks for their outgrowth, phenotypic properties, and the expression of the following viral proteins: latent membrane protein (LMP-1) and the EBV nuclear antigen complex (EBNA). Our study shows that IL-15 has the ability to control EBV-infected/transformed cells. Western blot and immunofluoresence analysis carried out three weeks post-infection showed no detectable levels of LMP-1 and EBNA expression in the IL-15 treated cultures. Phenotypic studies performed over this period showed that IL-15 specifically induces the expansion of CD3+CD56+ T lymphocytes with high percentage of the cells coexpressing CD8 antigen. No specific proliferative effect of IL-15 on the CD3-CD16+ NK cells was observed during this study. These findings ascertain the role of IL-15 in the induction of non-specific immune response that might be mediated by the CD3+CD56+ subset of cells, which in turn would inhibits the outgrowth of the EBV-infected/immortalized cells.

INTRODUCTION

The Epstein-Barr virus (EBV) is a ubiquitous human lymphotropic gamma herpesvirus (1), which has been the subject of extensive studies due to its association with various human pathologies. EBV is the etiological agent of infectious mononucleosis (IM) (2), a self limiting lymphoproliferative illness, and has been linked to a growing list of human malignancies including endemic Burkitt's lymphoma (BL) (3), Hodgkin's disease (HD) (4), immunoblastic lymphoma in immunosuppressed individuals (5), undifferentiated nasopharyngeal carcinoma (6), various T cell lymphomas (7, 8), salivary gland tumors (9), and acquired immunodeficiency-associated non-Hodgkin lymphoma (10). Following primary infection, EBV persists for life in a subset of B cells mainly in a latent form (11). EBV is the only known human lymphotropic virus that has the ability to induce and maintain the proliferation of infected human B-lymphocytes both *in vivo* and *in vitro* (12). The EBV genome persists in infected cells either as an episome or as an integrated viral DNA, with only a limited number of EBV genes being expressed in the latently infected cell (13). The latent gene products include six nuclear antigens, EBNA-1, -2, and –3a, -3b, -3c, and LP, and three latent membrane proteins, LMP-1, -2A, and –2B. Some of these latent proteins play a key role in the establishment and maintenance of the transformed state (14). The LMP-1 and EBNA-2 proteins are known to be potent gene transactivators that can induce cellular changes consistent with cellular activation and growth (15).

Primary infection with EBV leading to IM is associated with a remarkable immune stimulation. One of the hallmarks of this stimulation is the activation and expansion of T lymphocytes with cytotoxic and suppressor effector functions (16, 17). Although there is a strong humoral response to EBV, it is the cellular immune response that is believed to be primarily

responsible for controlling EBV infection (18). A number of effector mechanisms are likely to be involved which might be regulated by cytokines released from activated cells, as well as from accessory cells following their interaction with the virus (19). It is noteworthy that it has been shown since long that peripheral blood lymphocytes from individuals with EBV-induced IM display a remarkable non-specific cytotoxic activity (20, 21). We thus asked whether IL-15, a recently recognized cytokine with potent enhancing effect on non-specific lymphocyte cytocidal activity (22), would display any such activity against EBV-infected/transformed cells *in vitro*.

IL-15 was originally isolated from the supernatants of the simian kidney epithelial cell line CV-1/EBNA in 1994 (22). Despite a lack of amino acid sequence homology, IL-15 has similar tertiary structure and shares many of the biological activities with IL-2. These shared activities stem from the common use of the β- and γ-chains of the IL-2 receptor (IL-2R) by IL-15 and IL-2 for binding and signal transduction (23). In vitro analyses have shown that IL-15 is a T cell chemoattractant (24). IL-15 also induces proliferation of both native and phytohemagglutinin-stimulated human PBMCs (22, 25), stimulates B cell proliferation and differentiation (26) and inhibits apoptosis *in vitro* and *in vivo* (27). In addition, IL-15 acts as a costimulator with IL-12 to facilitate the production of IFN-γ and TNF-α from NK cells (28), and promotes the induction of cytolytic effector cells, including cytotoxic T cells and lymphokine-activated killer cells (LAK) (22). Although IL-15 shares biological activities with IL-2, there are several properties of IL-15 distinct from those of IL-2. IL-15 uses a specific α-chain of the IL-15R other than the α-chain of the IL-2R (29). In addition, while IL-2 is selectively expressed in activated T cells, IL-15 mRNA has been found to be constitutively expressed in several human tissues including: epithelial cells, placenta, skeletal muscle, kidney, lung, heart, and activated monocytes/macrophages (22). This difference in the expression pattern of IL-2 and IL-15 suggests varied *in vivo*

roles for each of these cytokines.

A previous study from this laboratory demonstrated that infection of PBMC with the human herpesvirus-6 (HHV-6) results in the induction of IL-15 and enhancement of NK cell activity in these PBMC cultures (30). More recently, we found that IL-15 production by PBMC represents an early response of these cells to viral infection (31, 49). Given these observations, we attempted to investigate whether IL-15-mediated immune response represents an important anti-viral effector mechanism. We therefore chose to determine the effect of IL-15 on the outgrowth of EBV-infected/transformed cells in PBMC cultures *in vitro*. Our results show for the first time that addition of recombinant human IL-15 (IL-15) to EBV-infected PBMC cultures results in complete inhibition of the outgrowth of EBV-infected/transformed cells in these cultures.

MATERIALS AND METHODS

PBMC isolation

Blood was collected from healthy EBV-seropositive and -seronegative donors in heparinized tubes. EBV-seropositive and EBV–seronegative status of these donors was defined by the presence or absence, respectively, of antibodies to EBV capsid antigen (VCA), as well as to EBV nuclear antigen complex (EBNA) using immunofluorescence assay. PBMC were isolated by centrifugation of blood on Ficoll-Hypaqe (Pharmacia) gradient by standard procedure (30). After washing, cells were resuspended at a concentration of 1×10^6 cells/ml in complete medium composed of RPMI-1640 supplemented with 20% heat-inactivated fetal bovine serum (FBS) containing 100 IU/ml penicillin, 20 µg/ml streptomycin, 1.0 µg/ml gentamycin, and 1.0 % glutamine.

B cell purification

Blood was collected in tubes containing the sodium-citrate anticoagulant and PBMC were obtained as described above. B cells were recovered from PBMC by negative immunoselection using Stem Cell Purification System (Stem Cell Technologies, Vancouver, BC), according to the manufacturer's instructions. Briefly, 5×10^7 cells/ml were incubated with 100 µl/ml of a cocktail of monoclonal antibodies (mAbs) reactive against human glycophorin A, CD3, CD14, CD16, and CD56 antigens for 30 min on ice. Cells were then reincubated for an additional 30 min with 60µl/ml magnetic colloid on ice. Unlabeled cells were collected using magnetic cell

separator (Macs, Biotec, Germany). The purity of obtained cells was greater than 95% and less than 1% were T, NK, and monocytic cells, as determined by flow cytometry.

Cell lines and reagents

B95-8, K562, and BJAB cell lines were cultured at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat inactivated FBS and antibiotics. Human recombinant IL-15 and monoclonal antibodies to human IL-15 (M110, and M112) were a gift from Immunex (Seattle, WA). IL-15 was used at a concentration of 50 ng/ml, and anti IL-15 was used at a concentration of 10 μg/ml throughout the study. The effectiveness of anti-IL-15 on IL-15 was evaluated by its ability to neutralize the stimulatory effect of IL-15 on NK cell activity (data not shown, 30). Anti-LMP-1 monoclonal antibody (S12) was kindly provided by Dr. D. Thorley-Lawson (Tuffs University, Boston, Ma). Peridinin chlrophyll protein (PerCP)-conjugated anti-CD3, -CD4, fluorescein isothyocyanate (FITC)-conjugated anti-CD8, -CD16, and phycoerythrin (PE)-conjugated anti- CD56 mouse mAbs were all purchased from Becton Dickinson Immunocytometry Systems (Becton Dickinson, CA) and used according to the manufacturer's instructions.

EBV preparation, infection and cytokine treatment

The EBV used in these experiments was obtained from cell free supernatant of the EBV-producing B95-8 cell line as previously described (33). No PMA was added during EBV preparation. Aliquots of the virus were kept frozen at –80°C until used for B cell transformation *in vitro*. This viral preparation had a titer of 10^4 EBNA-inducing unite as determined using B-JAB cells as described (48).

For EBV infection and cytokine treatment, freshly isolated PBMC (1X 10^6 cells) were treated with 200 µl of viral suspension or mock-infected supernatant for 60 min at 37°C in 5% CO_2. After washing with RPMI-1640 medium supplemented with antibiotics, EBV- and mock-infected cells were resuspended in 1 ml of complete medium in the presence or absence of IL-15 (50 ng/ml), anti-IL-15 (10 µg/ml), or IL-15 plus anti-IL-15. The cells were then cultured into 96-well round-bottom microtiter plates (2X 10^5cells/well/200µl) and were refed twice weekly with complete medium supplemented as above.

Proliferation assay

Cellular proliferation was measured by adding 1µCi of [^{3}H] thymidine to cultured cells during the last 6 h of incubation at 37°C in 5% CO_2. Cells were then harvested on glass-fiber filter paper and the incorporation of radioactivity was determined in a liquid scintillation counter. Results represent the mean of four replicate wells ± SE and are expressed as counts per minute (cpm) of [^{3}H] thymidine incorporation.

Immunofluorescence analysis

The presence of specific surface antigens was determined by staining with mAbs using three-color flow cytometric analysis. Briefly, after washing with PBS, 2×10^5 cells were incubated with either a combination of CD3-perCP, CD16-FITC, CD56-PE or a combination of CD4-perCP, CD8-FITC, CD56-PE for 30 minutes at 4°C. Cells were washed with chilled PBS and fixed with paraformaldehyde-PBS (1%, v/v). The stained cells (1×10^4) were then analyzed for fluorescence within 24 h with a FACScan flow-cytometer (Becton Dickinson). IgG1 isotype conjugated to PE, FITC, and perCP were

also used as controls. All mAbs were used following manufacturer's instructions.

Western blot analysis

Three weeks post-infection, cells treated as described above, were analyzed for LMP-1 protein expression. Briefly, 2×10^6 cells were collected from each experimental condition, washed twice in PBS and resuspended in 50 µl of ice-cold suspension buffer (0.1M Nacl, 0.01M Tris pH 7.6, 1mM EDTA, 1 µg/ml Aprotinin, 100 µg/ml PMSF, 1 µg/ml Leupeptin) on ice. Fifty µl of 2X SDS gel loading buffer (100mM Tris.cl PH 6.8, 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added, vortexed for 20 sec, and boiled for 5 min. Then, the lysate was centrifuged at 12000 rpm for 10 min and the supernatant was resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electro-transferred to a PVDF membrane (Millipore, Bedford, MA.). Transfer efficiency was established by co-transfer of prestained protein marker (GIBCO BRL/Life Technologies, Burlington, Ont.). S12 mAb (1:2000) specific for LMP-1 protein and goat anti-mouse IgG coupled to horseradish peroxidase were used in immunoblotting assay. The immunoreactive bands were detected using enhanced chemiluminescence reagents (ECL, DuPont-NEN, Boston, MA) on X-ray film (Eastman Kodak, NY).

Indirect immunofluorescence

The anticomplement immunofluorescence test was carried out for EBNA detection as described by Reedman and Klein (34). Briefly, cell smears were air-dried at room temperature for 60 min and fixed in acetone-methanol (1:1, v/v) for 3 min at –20 °C. The fixed smears were then treated with heat-inactivated standard EBV-positive serum from a healthy individual for 60 min

at room temperature in a humidified chamber. Smears were then washed three times for 5 min each with PBS. Untreated serum (used as a source of complement), obtained from EBV-seronegative healthy donor was added to the smears and incubated at room temperature in a humidified chamber for 60 min. Following washing with PBS as above, cells were then stained with FITC-conjugated anti-human β1C/β1A goat serum for 60 min at room temperature in the dark. Smears were washed with PBS as above and mounted in PBS: glycerol (1:1, v/v). Stained preparations were examined under a fluorescence microscope.

Statistical analysis

The data are presented as the mean ± SE of experiments. Differences were analyzed for significance by student's *t*-test and a *p* value of less than 0.05 was taken as significant.

RESULTS

Effect of IL-15 on EBV-induced lymphocyte transformation in vitro

In order to investigate the effect of IL-15 on the EBV-induced immortalization in vitro, PBMC from seven EBV-seropositive and one EBV-seronegative healthy individuals were infected with the EBV-transforming strain (B95-8), and incubated in the presence or absence of IL-15 and/or anti IL-15 antibodies for 21 days as described. Microscopic analysis showed that while PBMC incubated without viral infection for 21 days did not show any sign of EBV-induced transformation (Fig. 1, a, upper panel), EBV infection of PBMC in vitro resulted in typical EBV-induced transformation clusters starting 15 days post-infection (Fig. 1, b, upper panel). However, incubation of EBV-infected PBMC in the presence of 50 ng/ml of IL-15 resulted in the absence of EBV-induced transformation clusters through 21 days post-infection (Fig. 1, c, upper panel). Adding a monoclonal antibody to IL-15 (10μ g/ml) to the EBV-infected PBMC cultured in the presence of IL-15 resulted in the observation of typical EBV-induced transformation clusters, thus demonstrating the specificity of IL-15's effect on the EBV-induced transformation (Fig. 1, d, upper panel).

Immunofluorescence analysis for detecting EBV nuclear antigen (EBNA) expression in these PBMC cultures using a reference serum from an EBV-seropositive donor supported our microscopic results. As shown in the figure 1, while PBMC incubated without viral infection showed 0% EBNA positivity (Fig. 1, a, lower panel), *in vitro* infection of PBMC with EBV resulted in 90% EBNA positivity 21 days post-infection (Fig. 1, b, lower panel). However, EBV-infected PBMC incubated for similar period of time in the presence of IL-15 showed 0% EBNA positivity (Fig. 1, c, lower panel).

Incubation of EBV-infected PBMC in the presence of neutralized IL-15 resulted in 65% EBNA positivity (Fig. 1, d, lower panel). Identical results were obtained in all the experiments carried out using PBMC from eight different individuals (data not shown).

Immunoblotting analysis was also carried out to determine LMP-1 protein expression in these cultures from EBV-seronegative and EBV-seropositive donor 21 days post-infection and cytokine treatment. As shown in Fig. 2, LMP-1 protein that was detected in EBV-infected cells (lane #4) was absent when EBV-infected cells were cultured in the presence of IL-15 for 21 days (lane #5). Furthermore, LMP-1 protein band was detected with a lesser intensity from cells cultured in the presence of IL-15 plus anti-IL-15 antibodies (lane #6) as compared to EBV-infected cells (lane #4). No band was detected from PBMC cultured without any treatment (lane #3). The EBV-negative cell line, K562 (lane #1) and EBV-positive cell line, LCL (lane #2) were used as negative and positive controls, respectively. Taken together, these data clearly demonstrate that the presence of IL-15 resulted in the inhibition of EBV-transformed cells in PBMC cultures *in vitro*.

We then examined whether IL-15 treatment was effective against EBV-transformed cells at later stages of infection as well. For this purpose, PBMC from an EBV-seronegative individual were infected with EBV or treated with mock-infected supernatant, as described in Materials and Methods. Two weeks later, when the EBV-induced immortalization of B lymphocytes was confirmed by microscopic observation of the cellular outgrowth and EBNA antigen expression in EBV-infected cultures (80% $EBNA^{+}$), EBV-infected and mock-treated cells were then cultured in the presence or absence of IL-15 for additional 10 days. The effect of IL-15 on the EBV-induced transformation in the cultured cells was then determined by detecting EBNA antigen expression by immunofluorescence assay. As

illustrated in Fig. 3, EBNA antigen-expressing cells from these EBV-infected PBMC were eliminated when infected cells were cultured in the presence of IL-15 for 10 days. In contradistinction, EBV-infected PBMC incubated in the absence of IL-15 showed 93% EBNA positivity after incubation for similar period of time. Mock-treated, control PBMC cultured in the presence or absence of IL-15 showed 0% EBNA positivity after 24 days of incubation. The mortality of PBMC cultured in the absence of EBV infection and IL-15 was found increased up to 35% as compared to the EBV-infected and/or the cytokine-treated cells.

Effect of IL-15 on EBNA expression in B95-8 cell line and on purified fresh B lymphocytes

In order to determine whether IL-15 has any direct inhibitory effect on EBNA synthesis, cells of the EBV-producer B95-8 cell line, which contain multiple copies of EBV genome, were cultured for several days in the presence or absence of IL-15. Immunofluorescence analysis indicated no significant change in EBNA expression in these cells (data not shown). In addition, the presence of IL-15 had no significant effect on the growth or viability of B95-8 cells.

Since B cells are known to be the principal targets for EBV immortalization, we examined whether IL-15 has a direct inhibitory effect on freshly isolated B cells in the presence or absence of EBV infection. B cells were purified from freshly isolated PBMC from EBV-seronegative and EBV-seropositive individuals using negative selection procedure as described. These purified B cells were then infected with EBV or treated with mock-infected supernatant as described above. Cells were then cultured in the presence or absence of IL-15 and/or anti-IL-15 antibodies and examined for appearance of EBV-induced transformed cluster cells microscopically. The

transformed status of the EBV-infected B cells in these cultures was confirmed by EBNA expression (data not shown). As illustrated in Fig. 4, IL-15 treatment for 15 days significantly induced B cell proliferation in the presence or absence of EBV infection as compared to the B cells cultured without IL-15. The effect of IL-15 treatment on B-cell proliferation was abolished by neutralization with a monoclonal antibody to IL-15. Our data suggest that IL-15 has no direct inhibitory effect on B cells in the presence or absence of EBV infection.

Effect of IL-15 on PBMC proliferation

In Preliminary experiments, IL-15 was found to induce proliferation in PBMC cultures as early as 5 days post-treatment with this cytokine (data not shown). To identify the cell population undergoing this proliferation, freshly isolated PBMC from EBV-seronegative and EBV-seropositive individuals were infected and cultured in the presence or absence of IL-15 or IL-15 plus anti IL-15 antibodies as described above. Three color flow cytometric analysis using a combination of labeled monoclonal antibodies against CD3, CD16, and CD56 antigens was carried out on cell samples prior to the addition of IL-15 and on 7, 14, and 21 days post IL-15 treatment. As shown in the figure 5 (A), IL-15 treatment for 21 days in the presence or absence of EBV infection *in vitro* induced the proliferation of CD3+CD56+ T cell subpopulation from 2.37% to 55.5% and to 59.3%, respectively. The percentage of CD3+CD56+ T cells did not change significantly in uninfected PBMC, EBV-infected PBMC and EBV-infected PBMC cultured in the presence of neutralized IL-15. In contrast, no specific proliferative effect of IL-15 was observed on the CD3-CD16+ NK cell population during the same period of incubation (Fig. 5, B); in fact, the percentage of CD3-CD16+ NK cells decreased from 7.47% to 0% in the EBV-infected PBMC 21 days post-treatment. The above results indicate that IL-15 treatment for 21 days induces the proliferation of the CD3+CD56+

T cell population in the presence or absence of EBV infection but not CD3-CD16+ NK cell population.

Furthermore, FACS analysis data showed that the increase occurred in the CD3+CD56+ T cell population in the presence of IL-15 treatment during 21 days of incubation was accompanied by a significant decrease in the CD3+CD56- T cell population. As shown in Fig. 6, the percentage of CD3+CD56- T cell population in the presence of IL-15 decreased from 59.0% at day one to 30.2% and 30.6% at day 21 in the presence or absence of EBV infection, respectively. Relatively, no significant changes were noted 21 days post-treatment in the percentage of the CD3+CD56- T cell population in the mock-infected or EBV-infected PBMC cultured in the absence of IL-15 cytokine.

Finally, in order to further characterize the CD3+CD56+ T cell population that proliferated in the presence of IL-15, three-color flow cytometric analysis using a combination of conjugated anti-CD4, -CD56, and -CD8 mAbs was carried out as described above. Fig. 7 represents data obtained from FACS analysis 21 days post-treatment. These results show that the majority of the CD3+CD56+ T cell that proliferated in the presence of IL-15 coexpress the CD8 antigen irrespective of the presence or absence of EBV infection (Fig. 7, h, and i, respectively) as compared to mock-infected or EBV-infected PBMC (Fig. 7, f, and g, respectively).

DISCUSSION

IL-15, a recently cloned cytokine (22), was first identified by its ability to support the growth of an IL-2-dependent cell line (CTLL). This cytokine was found to share many biological activities with IL-2 (22, 26, 28). However, IL-2 and IL-15 have different cellular sources. While IL-2 is exclusively secreted from T cells, IL-15 appears to be constitutively expressed in a variety of tissues, including activated monocytes/macrophages (22). The importance of IL-15 in the control of bacterial and parasitic infection have been recently demonstrated (35, 36, 37). However, its role in the immune response to viral infection has not yet been investigated. Two recent studies from our laboratory confirmed the induction of IL-15 in the supernatant of PBMC following viral infection in vitro (30, 31). Therefore, we hypothesized that IL-15 could have an important role in the immune control of acute viral infection. In the present study, we investigated the effect of IL-15 on EBV-induced lymphocyte transformation *in vitro*. Our results demonstrate for the first time that (a) addition of IL-15 to cultures containing EBV-infected PBMC abrogated the growth of EBV-transformed cells and that (b) IL-15 had no direct inhibitory effect on EBNA synthesis or on purified B cell growth. Our data also show that (c) IL-15 treatment of PBMC for 21 days specifically and significantly augmented the percentage of CD3+CD56+ T cell population that mainly coexpress CD8 antigen. The fact that IL-15 had similar effect on elimination of EBNA and LMP-1 expressing cells in EBV-infected PBMC cultures from both EBV-seropositive and EBV-seronegative healthy individuals, indicates that this effect was EBV-memory-independent. These results are in agreement with a previous study from our laboratory that described a similar EBV memory-independent inhibitory effect of IL-2 on EBV-induced transformation (38).

The results obtained from incubation of the EBV-producer B95-8 cell line in the presence or absence of IL-15 further demonstrated that IL-15's inhibitory effect on EBV-induced transformation is not a consequence of a direct effect of this cytokine on EBV gene expression. In addition, the significant proliferative effect on both mock-infected and EBV-infected purified B lymphocytes following 15 days of incubation in the presence of IL-15 as compared to control B lymphocytes further ascertain the absence of direct inhibitory effect on B lymphocytes *in vitro*. Recently, other investigators have shown the proliferative effect of IL-15 on pre-activated tonsil B cells (26), and freshly isolated leukemic B cells from patients with B-cell lymphoproliferative disorders (25). Nevertheless, these studies reported the absence of proliferative effect of IL-15 on normal resting B cells as tested *in vitro* following incubation of cultured cells with IL-15 for 3-5 days only. Our data show a significant proiferative effect of IL-15 on freshly purified B lymphocytes following incubation with this cytokine for 15 days; it is noteworthy that we did not observe any significant proliferative effect on resting B cells 5 days post IL-15 treatment as compared to control B lymphocytes (data not shown).

The elimination of EBV-transformed cells in PBMC cultures following their incubation *in vitro* with IL-15 would suggest that IL-15 might also inhibit EBV-induced cellular outgrowth in *in vivo* situation. Previous studies have shown that IL-15 specifically induces the proliferation of T cells (35, 36). In a recent study, Jullien et al. (37) reported that the presence of a large CD3+CD56+ T cell population in the lesions of the self-healing form of *M. leprae* infection was associated with the presence of secreted IL-15. The expansion of highly cytotoxic effector cells with both the T cell marker CD3 and the NK cell marker CD56 from freshly isolated PBMC has been previously reported; Schmidt-Wolf et al., (39) reported a protocol for generation of these cells by culturing PBMC in the presence of IFN-γ on day

1, and in the presence of rIL-2, mAb against CD3, and IL-1α the following day. CD3+CD56+ Cytotoxic effector cells were termed cytokine-induced killer cells (CIK) and observed to have a superior *in vivo* antitumor activity as compared to the lymphokine-activated killer (LAK) cells and more accessibility to the tumur site than tumor-infiltrating lymphocytes (TIL) (40). Later study has shown that these cells mediate highly efficient MHC non-restricted killing of tumor cell targets (41). Recently, CIK cells were shown to kill target cells in a granular-dependent cytolysis manner (42). In our study, we have shown that treatment of freshly obtained PBMC for 21 days with IL-15 alone can induce the generation of CD3+CD56+ T lymphocytes. Previous studies (40, 41, 43) showed that the expanded CD3+CD56+ T cell lymphocytes originated from the CD3+CD56- T cell population and not from CD3-CD56+ NK cell population, our present data would support these observations.

On the other hand, several studies have shown the ability of IL-15 to induce NK cell activity in short-term incubation period (72-84 h) (22, 28, 30, 31, 32, 49). However, the proliferative effect of IL-15 on NK cell population in long term incubation has not been well demonstrated. Our FACS analysis data demonstrate that long term incubation (21 days) of PBMC with IL-15 did not significantly induce CD3-CD16+ NK cell proliferation (fig.5, b). Nevertheless, in the presence of IL-15, CD3-CD16+ NK cell population was found to survive in cell cultures for 21 days. This observation is consistent with a recent study by Carson et al., (44) in which the ability of IL-15 to function as a survival factor for the NK cell population was reported.

IL-2 has been shown to have remarkable therapeutic benefits against some cancers (45); however, this was found not to be without cost as *in vivo* IL-2 administration is associated with substantial dose limiting toxicity known as vascular leak syndrom (46). Interestingly, IL-15 has been shown to have

overlapping biological activities with IL-2, and *in vivo* analysis using animal model has shown that IL-15 mimics the antitumor activities of IL-2 with potentially less toxicity (47). From these observations and the results presented above, it is tempting to suggest that IL-15 may be a suitable candidate for elaboration of a cytokine-based immunotherapeutic approach aimed at the eradication of EBV-infected/transformed cells in affected patients.

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FIGURES LEGENDS

Figure 1: Effect of IL-15 on EBV-induced transformation. Freshly isolated PBMC from 8 healthy donors were collected as described in Materials and Methods. 1×10^6 cells were infected with the B95-8 EBV strain, and cultured in 96 well-plates (2×10^5 cells/well/200μl) in the presence or absence of IL-15 (50ng/ml) and IL-15 plus anti-IL-15 (10μg/ml) for 21 days. Following incubation, cells were collected and assessed for cellular outgrowth (a, b, c, d, upper panels) and EBNAs synthesis (a, b, c, d, lower panels). A culture was considered to have EBV-induced transformation when it revealed more than 50% EBNAs positivity. (a) PBMC, (b) EBV-infected PBMC, (c) EBV-infected PBMC in the presence of IL-15, (d) EBV-infected PBMC in the presence of both IL-15 and anti-IL-15. Magnification, × 10 for upper panels, and × 40 for lower panels.

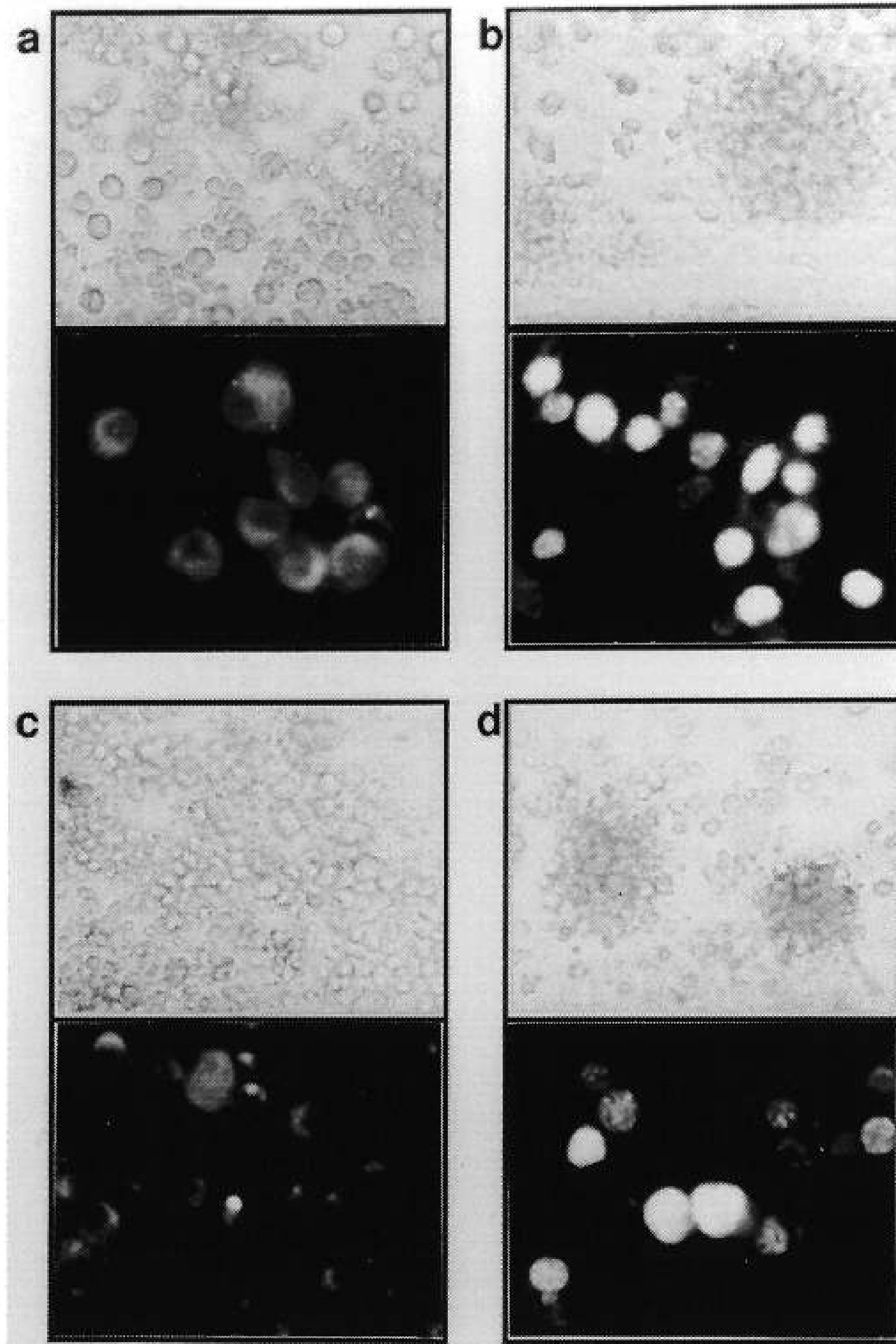

Figure 1: Effect of IL-15 on EBV-induced transformation

Figure 2: Effect of IL-15 on LMP-expression following EBV infection of PBMC. Twenty-one days post-infection and treatment as in figure 1, cells were assayed for LMP-1 protein synthesis. 1×10^6 cells were washed, lysed, and boiled for 5 min. The lysates were resolved by SDS-PAGE, and immunoblotted with LMP-1 specific mAb. The migration positions of m.w. marker are indicated in Kd. Results shown from cells of an EBV-seronegative individual and representative of two independent experiments. Lane 1: K562, EBV-negative cell line; lane 2: LCL, EBV-positive cell line; lane 3: untreated PBMC; lane 4: EBV-infected PBMC; lane 5: EBV-infected PBMC in the presence of IL-15; lane 6: EBV-infected PBMC in the presence of both IL-15 and anti-IL-15.

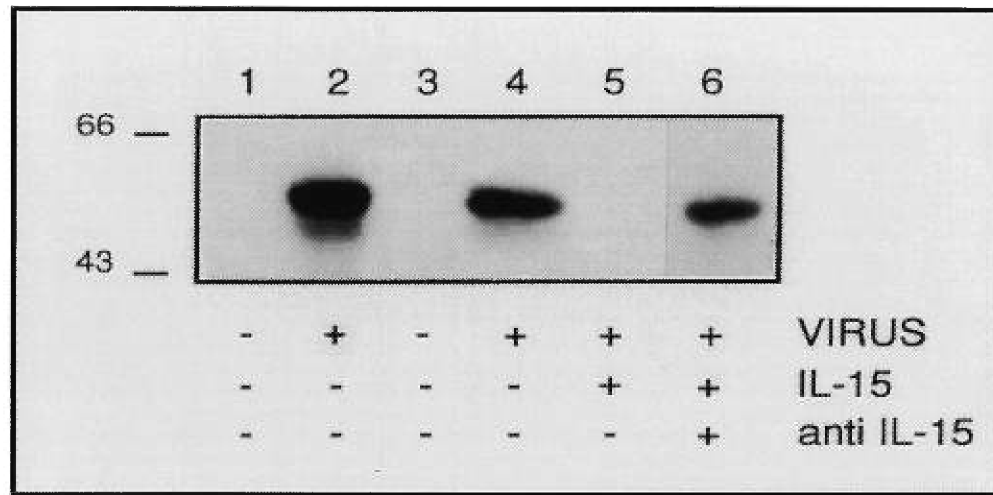

Figure 2: Effect of IL-15 on LMP-expression following EBV infection of PBMC

Figure 3: Effect of IL-15 on the growth and EBNAs synthesis in freshly transformed cells. PBMC (1×10^6) from EBV-seronegative healthy individual were treated with EBV or mock-infected supernatant as described. Cells (2×10^5 cells/well/200μl) were cultured in complete medium till the EBV-induced transformation was assured in the EBV-infected PBMC by EBNAs synthesis. Then, cultured PBMCs were incubated in the presence or absence of IL-15 for additional 10 day. The effect of IL-15 was evaluated by EBNAs synthesis and the mortality was determined by trypan blue staining.

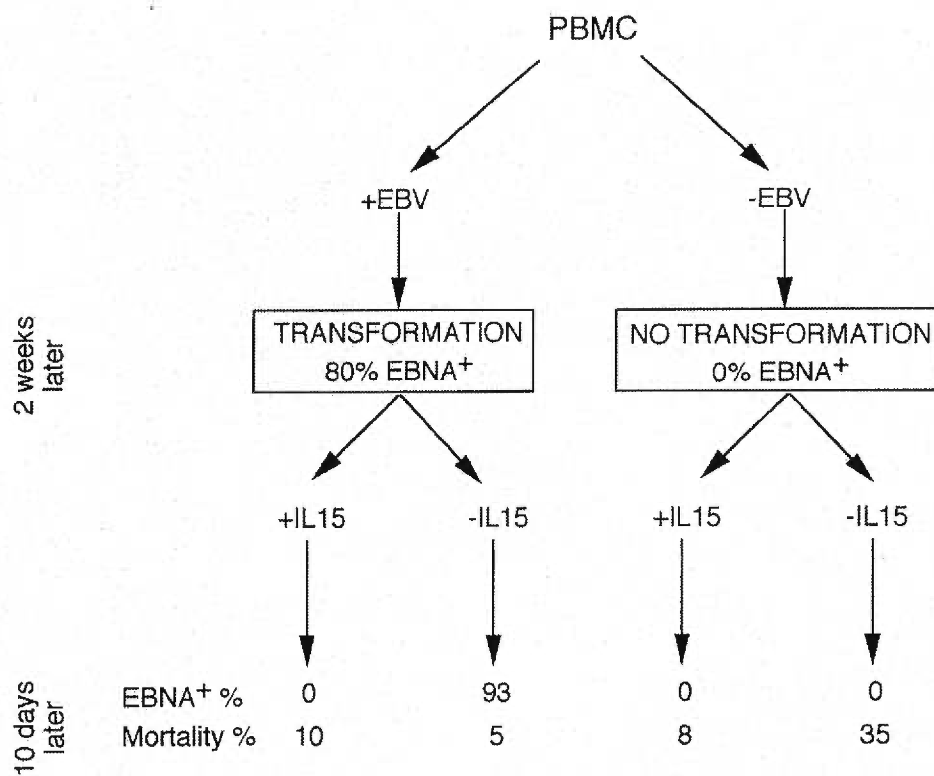

Figure 3: Effect of IL-15 on the growth and EBNAs synthesis in freshly transformed cells

Figure 4: Effect of IL-15 on the growth of purified B cells in the presence or absence of EBV. PBMC from EBV-seropositive and EBV-seronegative were first isolated and then B cells were purified by negative selection as described in Materials and Methods. Purified B cells were then infected with EBV or treated with mock-infected supernatant, and cultured (2×10^5 cells/well/200μl) in the presence or absence of IL-15, anti-IL-15 and IL-15 plus anti-IL-15. When the EBV-induced transformation in the infected B cells was assured by EBNAs positivity, the proliferation response was determined by [^{3}H] thymidine incorporation. Results represent the mean of four replicate wells ± SE and are expressed as counts per minute.

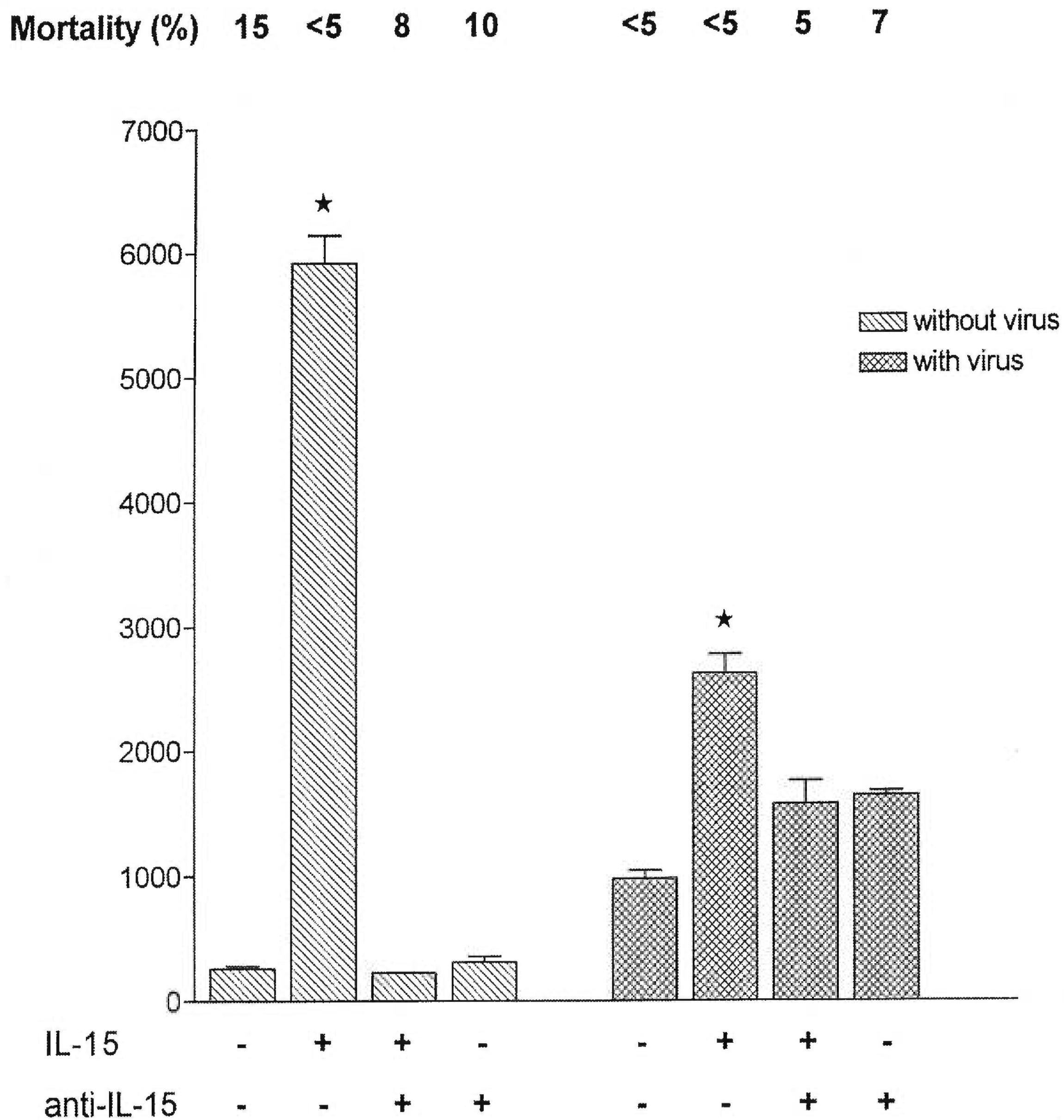

Figure 4: Effect of IL-15 on the growth of purified B cells in the presence or absence of EBV

Figure 5: FACS analysis of IL-15-treated PBMC cultures in the presence or absence of EBV infection. 1×10^6 PBMCs were infected and cultured cultured (2×10^5 cells/well/200µl) at 37°C, 5% CO_2 over 21 days in the presence or absence of IL-15 and/or anti-IL-15. Three color flow cytometric analyses using a combination of perCP-conjugated anti-CD3, FITC-conjugated anti-CD16, and PE-conjugated anti-CD56 were done on day 1 (pre-treatment), and 7, 14, 21 post-treatment. A) Effect of IL-15 on CD3+CD56+ T cell population; B) Effect of IL-15 on CD3-CD16+ NK cell population. Data expressed as % of fluorescence from EBV-seronegative individuals, and auto fluorescence were controlled with perCP-conjugated, FITC-conjugated, and PE-conjugated isotype match Abs. Similar results were observed from three independent experiments.

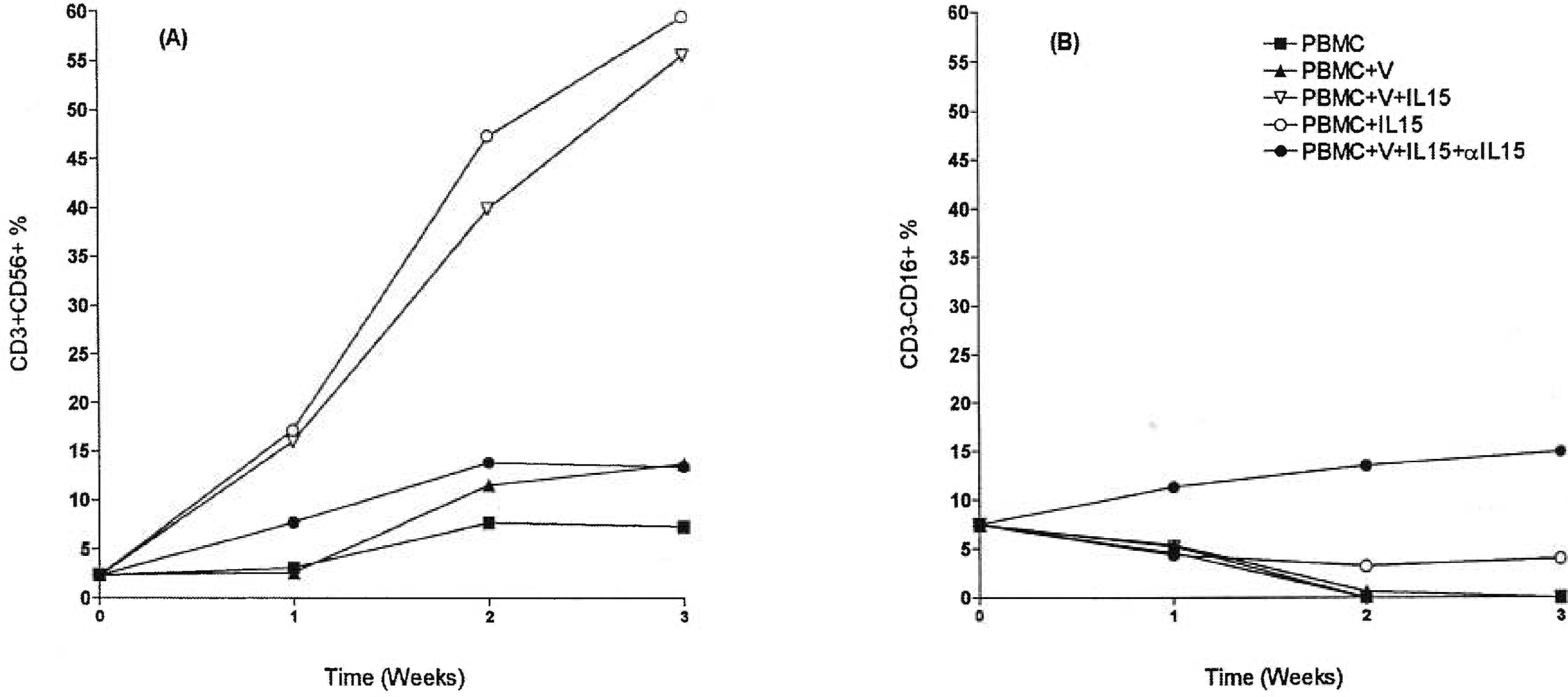

Figure 5: FACS analysis of IL-15-treated PBMC cultures in the presence or absence of EBV infection

Figure 6: **Effect of IL-15 on the percentage of CD3+CD56- T cell subset in PBMC in culture.** 1×10^6 cells were infected, treated, and cultured as in figure 5 over 21 days. Three color flow cytometric analyses using a combination of perCP-conjugated anti-CD3, FITC-conjugated anti-CD16, and PE-conjugated anti-CD56 were done on day 1 (pre-treatment), and 7, 14, 21 post-treatment. Data expressed as % of fluorescence from EBV-seronegative individuals.

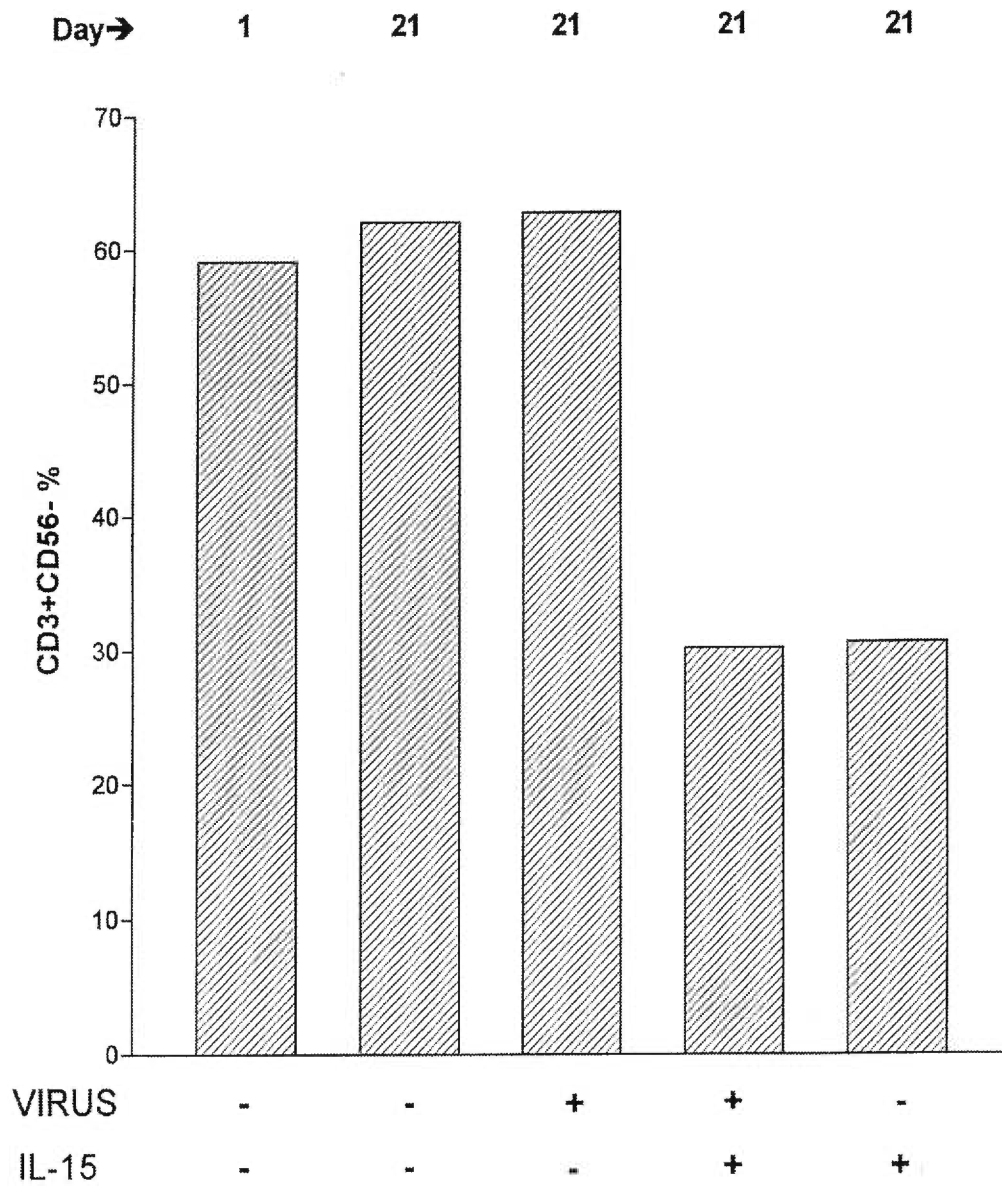

Figure 6: Effect of IL-15 on the percentage of CD3+CD56- T cell subset in PBMC in culture

Figure 7: FACS analysis of IL-15-treated PBMC cultures in the presence or absence of EBV infection. 1×10^6 cells were infected, treated, and cultured as in figure 5 over 21 days. Three color flow cytometric analyses using a combination of perCP-conjugated anti-CD4, FITC-conjugated anti-CD8, and PE-conjugated anti-CD56 were done on day 1 (pre-treatment), and 7, 14, 21 post-treatment. Data shown are from 21 days old culture of EBV-seronegative individual. The number in the upper right area represents the percentage of CD8+CD56+ or CD4+CD56+ T cell subset in the cultured PBMCs after 21 days of treatment. a, f) untreated PBMCs; b, g) EBV-infected PBMCs; c, h) EBV-infected PBMCs in the presence of IL-15; d, i) IL-15-treated PBMCs; e, j) EBV-infected PBMCs in the presence of IL-15 plus anti-IL-15. Similar results were observed from two independent experiments.

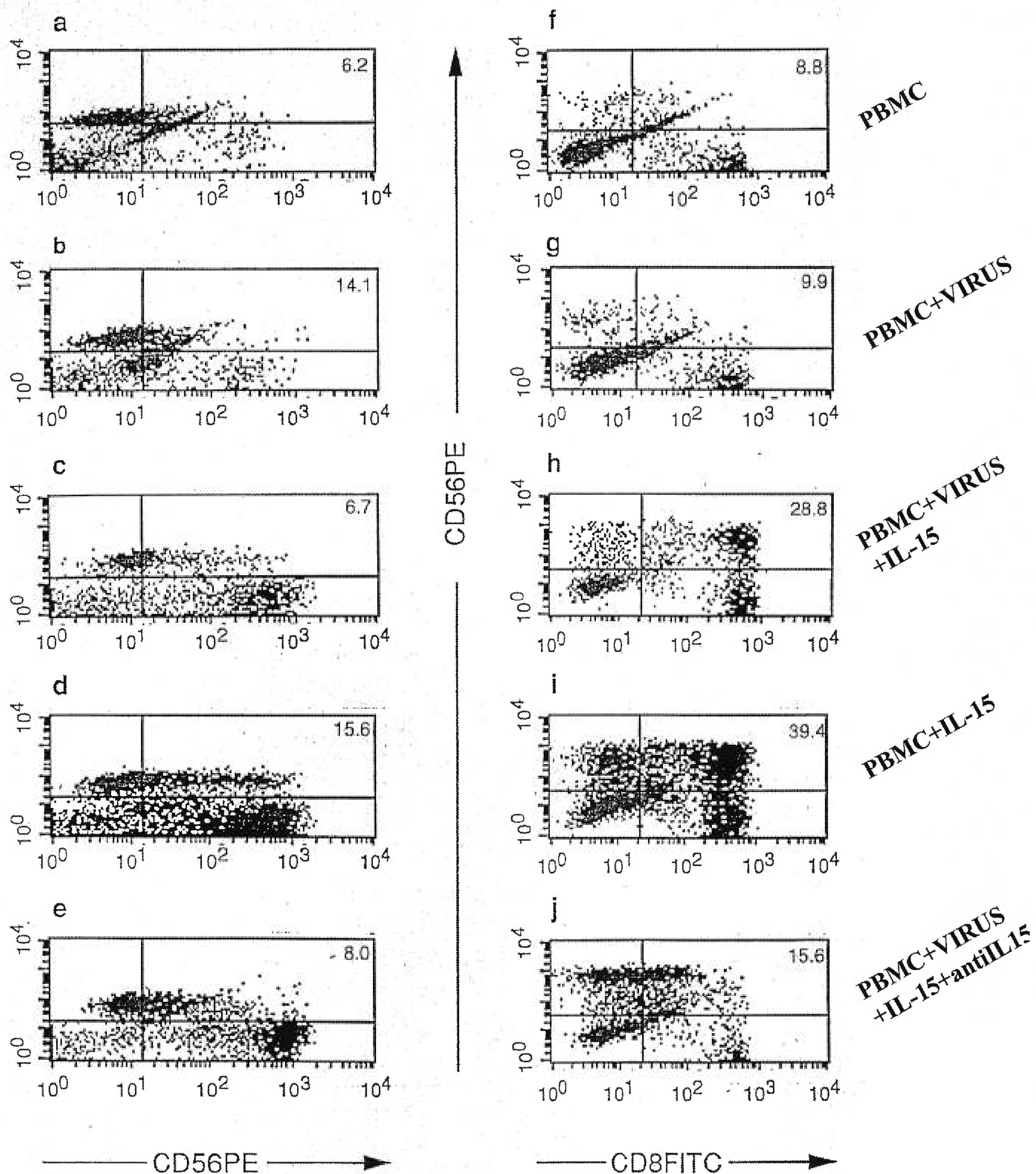

Figure 7: FACS analysis of IL-15-treated PBMC cultures in the presence or absence of EBV infection

III. GENERAL DISCUSSION

Earlier studies regarding the preferential lysis of EBV-infected cells led to the concept of the lymphocyte detected membrane antigen (LYDMA) expressed on B cells following EBV infection rendering them sensitive to recognition and lysis by immune T cells, that was postulated by Svedmyr and Jondal (1975) (Svedmyr and Jondal, 1975). However, the observation of LYDMA was challenged by several studies in which researchers provided evidence that the CTLs from IM patients were not specifically directed against an EBV-related membrane antigen (Klein et al., 1981; Rickinson et al., 1986; Patel et al., 1982; Sullivan, 1987). Rather, these studies revealed the importance of NK and non-specific T-cells. Therefore, in addition to the specific MHC-restricted CTLs which play a major role in controlling persistent EBV reactivation and proliferation, other non-specific memory-independent mechanisms exist that cooperate to generate an effective immune response. In between, cytokines play a major role either directly or indirectly by mediating differentiation, proliferation, and/or activation of the various components of the immune system. Important lessons regarding the role of cytokines in vitro and in vivo have been learned from models of infectious diseases.

The importance of cytokines in the activation of the immune system in response to invading organisms is well documented. IL-15 is a recently identified cytokine belonging to the four α-helix bundle cytokine families. Although IL-15 shares many biological activities with IL-2, there are several properties of IL-15 distinct from those of IL-2 that suggest unique *in vivo* roles for IL-15.

A previous report from our laboratory reported the ability of HHV-6 virus to induce IL-15 secretion from infected PBMC (Flamand et al., 1996). More recently, a study done by Fawaz et al (Fawaz et al., manuscript in

preparation) showed that IL-15 secretion and induction of NK cytotoxicity in response to viral infection represents a natural antiviral mechanism aimed to control and eliminates viral infection. Therefore, the present study was undertaken to investigate the proliferative effect of IL-15 in long term in vitro incubation with PBMC and to study the immunological function of this cytokine following EBV infection.

Results obtained from this study showed for the first time that EBV-infected/transformed cells were completely eliminated in the presence of IL-15 in the culture. Moreover, results observed from EBV-seropositive and EBV-seronegative individuals demonstrate that the inhibitory function of IL-15 is memory- independent. This is another feature added to the similarities between IL-15 and IL-2 in terms of the ability to mount an immune response against viral infection. A previous study from our lab demonstrated that EBV-induced cellular transformation was inhibited when IL-2 was added to the EBV-infected PBMC cultures (Khyatti et al., 1993).

IL-15 was seen to exhibit no direct inhibitory effect on neither the EBV genome nor on B cell growth. Indeed, results gathered from proliferation assays showed that IL-15 induced the proliferation of purified B cells. This proliferative effect was seen in the presence or absence of EBV infection. The data is in part in agreement with other reports published recently. Armitage, et al. (1995) showed that IL-15 significantly costimulates the proliferation and differentiation of purified tonsil B cells pre-activated with either anti-IgM or phorbol ester, but has no stimulatory effect on resting B cells (Armitage et al., 1995). Similar results were obtained by Trentin, et al. (1996) through examination of the effect of IL-15 on B cells collected from patients with B-cell chronic lymphoproliferative disorder. The investigators found that IL-15 triggers the proliferation of freshly isolated leukemic B cells but not normal resting B-lymphocytes (Trentin et al., 1996). The major discrepancy in the

results obtained from these two studies and our study in terms of IL-15's effect on normal resting B cells, can be explained by the differences in the incubation periods of IL-15 with the cultured cells. While the incubation time of IL-15 in those studies varied from 3 to 5 days in B cell proliferation assays, we incubated purified B cells with IL-15 for 16 days. It is worth noting that we did not observe any proliferative effect of IL-15 five days post treatment in both EBV-infected and mock-infected purified B cells using proliferation assay (data not shown).

Since IL-15 had no direct inhibitory effect on EBV-induced transformation of PBMC, and newly established (lymphoblastoid cell lines) LCL were found previously to be relatively resistant to lysis by NK cells (Seeley et al., 1981), we suggested that cellular effectors other than NK cells might be involved in the cellular immune response to EBV infection. Several previous studies support our hypothesis.

T lymphocytes collected from patients with IM were found to be able to kill EBV genome-positive LCL after depletion of FcR-positive NK cells (Bakacs et al., 1978; Galili et al., 1980). Other studies confirmed the cytotoxic activity of IM T lymphocytes and concluded that this activity was HLA independent (Lipinski et al., 1979; Seeley et al., 1981). In our experiments, a rapid and significant cellular proliferation occurred as early as 5 days following the addition of IL-15 to the cultured cells. Using several combinations of MoAbs and flow cytometry, the proliferated cells were found to be CD3+CD56+ T cells. The specificity of IL-15 proliferative effect on CD3+CD56+ T cells was ascertained through abrogation of the proliferation when a MoAb against IL-15 was added to the cultures. In addition, we found that the proliferative effect induced by IL-15 on the CD3+CD56+ T cells was accompanied with a significant decrease in the CD3+CD56- T cell subset following a similar period of incubation. This could be explained by the ability

of IL-15 to induce differentiation and proliferation of the CD3+CD56- T cells present in the PBMC culture. Data obtained from this experiment is consistent with previous findings whereby CD3+CD56+ T cells were expanded from CD3+CD56- T cells and not from CD3-CD56+ NK cells (Schmidt-Wolf et al., 1993; Lu and Negrin, 1994;Satoh et al., 1996). Moreover, FACS analysis performed on proliferated CD3+CD56+ T cells showed that the majority of the proliferated CD56+ T cells coexpressed CD8 a characteristic surface antigen of cytotoxic T lymphocytes. These data are in agreement with a previous report from Lu and Negrin (1994) where they demonstrated that the majority of the CD3+CD56+ killer cells originated from CD4-CD8+ T cells after culturing in the presence of IL-2 and a MoAb against CD3 for more than 1 month (Lu and Negrin, 1994).

Originally, the existence of T cells that mediate non-MHC-restricted cytotoxicity has been demonstrated by using an IL-2-dependent cytotoxic T cell clone that expresses the antigenic phenotype CD3+, NKH-1+ (CD56+) (Hercend et al., 1985). Nevertheless, the isolation of this T cell subset from normal peripheral blood suggested that these IL-2-dependent cytotoxic CD3+NKH-1 clones were not inadvertently antigen primed in vitro (Lanier et al., 1986). CD3 is a characteristic surface antigen expressed on T lymphocytes. CD56, originally known as the neural cell adhesion molecule (N-CAM) (Edelman, 1986), is a member of the Ig supergene family and is composed of five Ig-like domains and two fibronectin type III segments (Lanier et al. 1989). CD56 is predominantly expressed on NK cells and around 5% of peripheral blood T lymphocytes (Lanier et al., 1986). NK cells have been divided into two populations based on their CD56 expression. Approximately 10% of NK cells were found to express CD56 at high density ($CD56^{bright}$), while the majority expressed CD56 at low density ($CD56^{dim}$) (Ellis and Fisher, 1989). $CD56^{bright}$ NK cells are believed to be less differentiated than $CD56^{dim}$ cells due to their limited intrinsic cytotoxic

effector functionand constitutively express the high-affinity IL-2 receptor (Caligiuri et al., 1990; Matos et al., 1993). T lymphocytes expressing the CD56 antigen were found to be unique in their ability to mediate cell-mediated cytotoxicity against certain tumor cell targets in the absence of MHC restriction (Schmidt et al., 1986).

Recently, several studies have shown the importance of IL-15 in the stimulation of the immune response during primary infection. Jullien et al (1997) reported the role of IL-15 in the human immune response to an intracellular infection, *Mycobacterium leprae* (Jullien et al., 1997). The authors explained the immunoregulatory differences between two groups of patients with *M. leprae* infection, tuberculoid leprosy, and lepromatus leprosy. The former had a strong cellular mediated immune response against the pathogen and developed few lesions that tended to self-cure, whereas the latter group was not able to control the infection, which was progressive. Data gathered revealed that *M. leprae* induced IL-15 secretion from PBMCs of infected individuals. Nevertheless, the levels of both mRNA and IL-15 protein were found to be higher in patients with the self-healing form of the disease as compared to the patients suffering from the progressive type of the infection. In addition, the CD3+CD56+ T cell subset was expanded in lesions of the self-healing form of the infection and these cells were augmented specifically in response to IL-15 secreted in these lesions (Jullien et al., 1997). Another study by Nishimura et al published in 1996 showed that bacterial infections such as with *Salmonella choleraesuis* induces IL-15 secretion from infected macrophages (Nishimura et al., 1996). Data revealed that salmonella-infected macrophages expressed an abundant level of IL-15 mRNA, and the presence of IL-15 in the environment of the infected cells specifically stimulated the proliferation of the $\gamma\delta$ T cell subset and induced the production of γ-IFN from these proliferated cells. These studies are consistent with our findings and support the direct proliferative effect for IL-15 on CD56-expressing T

lymphocytes in vitro that is shown in this presented study.

Regarding NK cells and IL-15, as mentioned earlier, studies from our laboratory showed an upregulation of NK activity following viral infection of PBMC in vitro. Data gathered from these studies indicated that IL-15 was specifically responsible for the upregulation of NK activity. Nevertheless, non-of these studies analyzed the prolefirative effect of IL-15 on NK cell population. Data gathered from FACS analysis in the presented study demonstrate the absence of a significant proliferative effect of IL-15 on CD3-CD16+ NK cell subset following incubation of PBMC in the presence of this cytokine for 21 days. Interestingly, we observed a restoration of CD3-CD16+ NK cells within 21 days of incubation with IL-15. These data are consistent with a recent study published by Carson et al. (1997) in which the authors demonstrated the ability of Il-15 at picomolar concentrations to sustain resting human NK cell survival for up to 8 days in the absence of serum (Carson et al., 1997). This was explained by the ability of IL-15 to maintain Bcl-2 protein expression thus promoting resting lymphocyte survival.

The treatment of cancer patients represents a major therapeutic challenge. IL-15 has been shown in several in vitro studies to play an important role in antitumor immunity (Gamero et al., 1995; McInnes et al., 1996) and was proposed to be used in the design of a cytokine-based anti-tumor immunotherapeutic approach. One approach to treat patients with cancer is adoptive immunotherapy, a treatment in which immune cells with antitumor reactivity are transferred to the tumor-bearing host (Rosenberg, 1984). Previously, Gamero, et al (1995) reported the ability of IL-15 to induce LAK activity in PBMC from metastatic melanoma patients. Phenotypically, most of the LAK precursors and effector cells express surface NK markers, and are CD3-CD16+. These cells were able to kill both autologous melanoma tumor cells and LAK-sensitive tumor targets by a CD18-dependent, perforin-

related mechanism (Gamero et al., 1995). In addition, IL-15 was found to induce inflammatory cell recruitment *in vivo* following injection into the footpads of mice primed with *Corynebacterim parvum.* The majority of infiltrating cells was T cells and the infiltrate persisted for at least 3 days (McInnes et al., 1996). These cells are known as tumor-infiltrating lymphocytes (TIL), and have more potent antitumor activity than do LAK cells (Rosenberg et al., 1986). The majority of these cells are phenotypically CD3+CD56-CD8+ and are MHC-restricted (Rosenberg et al., 1986).

Regarding CD3+CD56+ T lymphocytes, a prior study has indicated that these cells can be expanded by culturing PBMC in the presence of IFN-γ on day 1, and in the presence of rIL-2, rIL-1α, and a MoAb against CD3 the following day. The majority of these cells were CD3+ and TCR-α/β+, and killed their targets through a non-MHC-restricted mechanism (Schmidt-Wolf et al. 1991). Cells expressing both the T cell marker CD3 and the NK marker CD56 were found to have the greatest cytotoxic activity (Schmidt-Wolf et al., 1993). For this reason, these cells were termed cytokine-induced killer (CIK) cells. These cells do not express the CD16 (Fc-receptor) surface molecule and, thus, do not participate in ADCC. Later, it was shown that CIK cells with the CD3+CD56+ phenotype had a superior in vivo antitumor activity in scid mice compared with that of LAK cells and were more readily accessible than TIL cells (Lu et Negrin, 1994). Mehta et al (1995) proposed two pathways by which CIK cells can kill target cells. The first pathway stimulated by the LFA-1 counterpart on CIK leads to a granular-dependent cytolysis. This pathway is sensitive to increased intracellular cAMP levels and is resistant to the immunosuppressive drugs CsA and FK506. The second pathway, which proceeds through stimulation of CD3 or CD3-like surface receptors on CIK cells, leads to granular-dependent cytolysis that is sensitive to increased intracellular cAMP levels, CsA and FK506 (Mehta et al., 1995). In our study, we showed that CD3+CD56+ T cells (CIK) rapidly proliferated following

incubation of normal human PBMC in the presence of IL-15 alone starting from the fifth day of culture.

Taken together, these studies as well as our own suggest that IL-15 would be a suitable candidate for the immunotherapy of cancer. Its preferential use over IL-2 stems from the dose-limiting toxicity associated with IL-2 administration that is known as vascular leak syndrome. In vitro studies that used a murine model revealed that the dose of simian IL-15 required to induce vascular leak syndrome in mice is approximately six times higher than that required for human IL-2 (Munger et al., 1995). On the other hand, IL-15 induces the expansion of CIK which are more efficient killer than LAK and TIL cells used in adoptive immunotherapy, especially that their use has faced several obstacles. Regarding LAK therapy, two major problems were noticed. First, is the inadequate tumoricidal activity of these MHC non-restricted polyclonal killer cells, and the second is the unavailability of sufficient numbers of activated effectors that retain both cytotoxic and tumor-targeting capability (Rosenberg et al., 1986). Problems encountered in TIL cell therapy are the small number of recovered cells from tumor biopsies, as well as the possibility that there may be alterations in function during extraction from human tissue (Whiteside, 1990). High doses of IL-2 treatment to circumvent these limitations have been faced with severe side effects (Rosnberg et al., 1989).

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VI. ANNEX

A COMPARATIVE STUDY OF THE UPREGULATION OF NATURAL KILLER CYTOTOXIC ACTIVITY VIA IL-15 INDUCTION BY DIFFERENT VIRUSES

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ABSTRACT

Interleukin (IL)-15 is a recently identified cytokine that belongs to the four α-helix bundle cytokine family and posseses biological activities similar to those of IL-2. Its ability to induce effectors of natural killer activity (NK) suggests its involvement during the innate immune response. In this respect, its role in the primary immune response to viral infection has only begun to be elucidated. In this study, we analyzed the effect of different viruses (Herpes simplex virus-1, Epstein-Barr virus, Respiratory Syncitial virus, Vesicular Stomatitis virus, Influenza virus, Reovirus and Sendai virus) on the upregulation of NK activity *in vitro*. We report here that the interaction of these viruses with peripheral blood mononuclear cells (PBMC) resulted in an immediate upregulation of NK activity via IL-15 induction; upon addition of monoclonal antibodies (mAbs) to IL-15, this effect was abrogated. Results of experiments carried out in parallel using monoclonal antibodies (mAbs) to IL-15 as well as to other cytokines (IL-2, IL-12, IFN-γ, TNF-α) clearly indicated that IL-15 was specifically responsible for the observed effect. Furthermore, supernatants of virus-infected PBMC from healthy donors significantly enhanced NK activity of uninfected PBMC *in vitro*. An increase of IL-15 protein levels twenty hours post-infection was also confirmed in a bioassay using an IL-2-dependent cell line CTLL. The kinetics of IL-15 mRNA expression were determined by a semi-quantitative RT-PCR; the level of expression of IL-15 messages peaked at different time points (4, 8 or 12 hrs) post-infection, depending on the nature of the virus. Taken together, these results suggest that the host's IL-15 response to viral infection and the subsequent NK cell activation represent an important effector mechanism of the host's innate immune surveillance against viral infections.

INTRODUCTION

IL-15 was first isolated and purified by Grabstein, et al in 1994 (1). The hIL-15 gene was mapped to chromosome 4 band 4q31. Several other human growth factors and cytokine genes map to the same arm of h-chromosome 4 including chemokines, the epidermal growth factor, the fibroblast growth factor, and IL-2 (2). Despite the absence of sequence homology, IL-15 and IL-2 are structurally similar and are both members of the short chain four helix bundle of cytokines (1). IL-15 utilizes both the β and γ chains of the IL-2 receptor for binding and signal transduction (3). Both IL-15 and IL-2 share the ability to support the growth of various T cell lines (1), antigen-dependent T cell clones (Tho, Th1, Th2) and activated normal T cells (5). They both have a costimulatory activity for the proliferation and Ig production of human tonsilar B cells, but IL-15 has no effect on resting B cells as tested in short term incubation *in vitro* (4). IL-15 is also an efficient activator of NK cytotoxic activity (5). IL-15 was found to activate human NK cells using components of the IL-2R and to synergize with IL-12 to significantly potentiate NK cell production of IFN-γ (6). Recent data from our laboratory suggested that IL-15 specifically induced the proliferation of CD3+CD56+ T lymphocytes in long term incubation of PBMC with IL-15 *in vitro* (45). Although IL-15 shares biological activities with IL-2, there are several properties of IL-15 distinct from those of IL-2. IL-15 uses a specific α-chain of the IL-15R -identified on a murine Th-2-cell clone- distinct from the α-chain of the IL-2R (36). In addition, while IL-2 is selectively expressed in activated T cells, IL-15 mRNA has been found to be constitutively expressed in several human tissues including: epithelial cells, placenta, skeletal muscle, kidney, lung, heart, and activated monocytes/macrophages (1).

NK cells are important effectors in the natural immune response, and

play a major role as the first line of defense against viral, bacterial and parasitic infections (53, 54). Through their non-MHC-restricted cytotoxicity, they can also eliminate tumor cells thus providing the host with a defense mechanism that would target intracellular pathogens as well as cancerous cells (59). Furthermore, NK cells act via their FcRIII receptor (CD16) as effectors in the antibody-dependent cell-mediated cytotoxicity (ADCC) (37). This allows the immune system to build an adaptive immune response aimed at eradicating the pathogen (55, 56). Therefore, any defect in NK function would leave the human host vulnerable to several major viral infections such as EBV (48) and HIV (49), hence undermining the body's immune response (57, 58).

Recent studies in this laboratory have shown that infection of human peripheral blood mononuclear cells (PBMC) with human herpesvirus-6 and human herpesvirus-7 resulted in an immediate upregulation of NK activity of these PBMC via IL-15 induction (7, 35). In light of the important regulatory role of cytokines in the initiation and maintenance of the immune response, and in order to gain a better understanding of the host's IL-15 response to viral infection as part of an innate defense mechanism, we sought to determine whether different, unrelated viruses have the ability to enhance NK activity via IL-15 induction of human PBMC following infection. Thus, we investigated the following viruses belonging to different families namely, the Influenza virus, Herpes Simplex Virus-1 (HSV-1), the Epstein-Barr virus (EBV), Reovirus, Vesicular Stomatitis virus (VSV), Sendai virus and the Respiratory Syncitial virus (RSV), for their ability to upregulate the natural killer cytotoxic activity of virus-infected PBMC *in vitro*. Here, we present data showing that all these viruses enhance the NK activity of infected PBMC and that this enhancement occurs within few hours of viral exposure and is specifically mediated via IL-15 induction. This induction was found to be at both the IL-15 mRNA and protein secretion levels in these infected PBMC. These findings suggest that IL-15 induction could represent an early effector mechanism of

the host's innate immune response to viral infection.

MATERIALS AND METHODS

Preparation and culture of PBMC

Heparinized venous blood, freshly obtained from healthy donors, was centrifuged over Ficoll-Hypaque (Pharmacia; Uppsala, Sweden) gradient, and PBMCs were collected as described (7). The separated PBMC were whashed, and cultured in RPMI-1640 culture medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1.0% glutamine, 100 U/ml penicillin, 20 µg/ml streptomycin, and 1 µg/ml gentamycin.

Cell treatment

The PBMC ($1x10^{6}$ cells) were infected with optimal doses of viruses (as determined in preliminary experiments for maximal induction of NK cytotoxicity), or treated with mock-infected culture supernatant for 2 h at 37ºC, washed with Hank's buffer (Gibco Laboratories), and then resuspended in 1 ml RPMI-1640 medium supplemented with 10% heat-inactivated FBS for 20 h at 37ºC with or without a monoclonal antibody to IL-15. After 20 h, cell-free supernatants from mock and virus-treated PBMC were collected and kept at -70ºC until used, and cells were prepared for the NK cytotoxicity assay as described (10).

Cell-free supernatants were added to untreated resting PBMC (24 h post separation) at 25% of the final volume, in the presence or absence of a mAb to IL-15 as well as mAbs to other cytokines such as IL-2, IL-12, IFN-γ, and TNF-•, before mixing with the NK cell targets K562 cells. The NK

activity of PBMC was tested in the presence of rhIL-15 (50 ng/ml) and anti-IL-15 (10 μg/ml).

Cell lines and viruses

K562 cell line was purchased from the American Type Culture Collection (ATCC) (Rockville, MD). CTLL-2 cell line was kindly provided from Dr. R-P Sékaly laboratory (IRCM, University of Montreal). K562 cells were cultured in RPMI-1640 supplemented with 10% FBS, and CTLL-2 cells were cultured in RPMI-1640 with 5% FBS supplemented with $5x10^{-5}$ M β-mercaptoethanol. The PBMC ($1x10^{6}$ cells) were infected with 200 μl of EBV (B95-8 strain) preparation with a titer of $2x10^{5}$ EBNA-inducing units/ml (13), 80 μl of HSV-1 at a multiplicity of infection of 50 PFU/cell (McIntyre strain) (13), 200 μl of hRSV (human respiratory syncytial virus) (Long strain) (ATCC) at a titer of 10^{7} SFU/ml (14), 200 μl of Sendai virus containing 750 HAU in PBS (15), 200 μl of Reovirus preparation (10^{8} PFU/ml) (kindly provided by Dr. Guy Lemay, University of Montreal), 50 μl of Influenza virus A (H1N1) strain A1/FM/1/47 with a titer of $10^{6.25}$ at a CEID 50/0.2 ml (ATCC), 200 μl of VSV 10^{-1} dilution 10^{5} TCID 50 (tissue culture infectious dose fifty) (ATCC). Viral preparations were first titrated to determine the dose that had the maximum NK-inducing activity (see below). Viral preparations were tested for the presence of endotoxin by the Limulus amebocyte assay (Sigma Chemical Co, St. Louis, Mo), and were found to contain <20 pg/ml of contaminating endotoxin.

NK cell cytotoxicity assay

All cytotoxicity assays were performed using either non-treated resting

PBMC, or virus-infected PBMC (20 h post-infection), as effectors. All assays were done using a standard ^{51}Cr-release assay as described (7). Briefly, K562 target cells ($1x10^6$ cells/ml) were labeled by incubation with 100 µCi of sodium chromate (^{51}Cr) (DuPont, Canada) for 1 h at 37 °C. The radiolabeled target cells were then washed four times with RPMI-1640, and resuspended at a concentration of $1x10^5$ cells/ml in RPMI-1640 with 10% FBS. K562 cells were then added into v-bottomed wells and mixed with 0.05 ml PBMC ($4x10^6$ cells/ml) from healthy donors at an effector: target (E: T) ratio of 20:1 and incubated for 16 h at 37 °C. Radioactivity was then measured using a gamma counter (LKB, Wallac model 1272, Finland). The percentage of cytotoxicity was calculated based on the following formula: (cpm experimental-cpm spontaneous)/(cpm maximum-cpm spontaneous) x100, where cpm is counts per minute. All these experiments were done in triplicate, and the results are presented as the mean ± standard error of three independent observations. The spontaneous cpm was determined by counting the radioactivity of the supernatant of the target cell suspensions, whereas the maximum cpm was determined from the radioactivity of the resuspended target cells with Triton X (100 µl).

Antibodies and Cytokines

Neutralizing mAbs to human IL-2, IL-12, and tumor necrosis factor-α (TNF-α) were purchased from R&D systems (Minneapolis, MN). Neutralizing mAb to interferon (IFN-γ) was purchased from Genzyme (Boston, MA). Human recombinant IL-15 and mAb to IL-15 were a gift from Immunex (Seattle, WA). All mAbs were used at a concentration of 10 µg/ml that was determined in a previous study in our laboratory (7), and IL-15 was used at a concentration of 50 ng/ml.

CTLL Assay

Supernatants of infected cells, obtained after 20 h incubation with the different viruses at a concentration of $2x10^6$ cells/ml were collected and tested for the presence of bioactive IL-15 by its ability to sustain the proliferation of the IL-2- and IL-15-responsive CTLL cell line (1). Treatment of supernatants with a mAb to IL-2 ascertained that proliferation of CTLL-2 cells was not due to IL-2 (data not shown). Cells were washed for three times and incubated in a 5% RPMI-1640 medium containing $5x10^{-5}$ M ß-mercaptoethanol for 3 h at 37ºC, in 5% CO_2. CTLL cells ($5x10^3$ cells/100µl) were then dispensed into 96-well round-bottom plates containing 100 µl of supernatant, and cultures were assayed in duplicates. After 66 h incubation at 37ºC in 5% CO2, 20 µl containing 1 µCi of [^{3}H] (Du Pont NEN, Boston, MA) was added to each well and the cultures were pulsed for an additional 6 h. Cells were then harvested on glass-fiber filter paper by an automated sample cell harvester (Tomtec) and dried. The incorporation of radioactivity was determined in a liquid scintillation counter. Results represent the mean of two replicate wells of two independent experiments and are expressed as counts per minute (cpm) of [^{3}H] thymidine incorporation.

Preparation of IL-15 mRNA, and RT-PCR

PBMC ($1x10^6$ cells) were treated with different viruses for 2 h at 37ºC, washed and resuspended in 1 ml RPMI-1640 supplemented with 10 % FBS. At various time intervals (2, 4, 8, 12 and 20 h) post-treatment, cells were counted and checked for mortality by trypan blue exclusion staining (< 5%), and $5x10^5$ cells were lysed and stored at -70ºC until assayed for IL-15 mRNA expression.

Total mRNA was extracted from cells using a modified guanidium isothiocyanate procedure as described (12). All reagents used were purchased from GIBCO BRL (GIBCO, BRL, Life Technologies, Ontario) unless indicated. Total RNA extracted was finally resuspended in a total volume of 40µl. ddH2O. The samples were then treated with 10 U DNase I for 30 min at 37ºC. The isolated RNA from infected and mock-treated PBMC was subjected to RT-PCR to determine the level of expression of IL-15 mRNA.

For first-strand cDNA synthesis, 4 µl of total RNA was reverse-transcribed in a total volume of 10 µl containing: 100 U recombinant moloney murine leukemia virus M-MLV reverse transcriptase, 2 µl 5x first strand buffer [250 mM Tris-HCl, 375 mM KCl, and 15 mM $MgCl_2$ (pH=8.32)], 1 µl of random hexamer primer (Pd N6), 0.01 M DTT, 0.5 µl 5 mM dNTP, and 30 U RNase inhibitor_(Pharmacia, Sweden). Following denaturation for 10 min at 65ºC, RNA was reverse-transcribed for 1 h at 42ºC, and then the RT enzyme was inactivated by incubation at 95ºC for 5 min.

PCR was performed with an aliquot of 5 µl of synthesized cDNA product in a reaction mixture containing 5 µl sscDNA, 5µl 10× PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 3 mM MgCl2, 50 pmole A and B oligonucleotide primers, 1 µl 5 mM dNTP, 2.5 U Taq polymerase, and distilled water to a total volume of 50 µl. The conditions were as follows: 3 min denaturation at 94ºC, 5 min annealing at 50ºC and 5 min extension at 72ºC for the first cycle; and denaturation for 1 min at 94ºC, annealing for 1 min at 55ºC and extension for 1 min at 72ºC for the subsequent cycles. The cDNA was then amplified in a DNA mini cycler (AECL, Chalk River. Ontario, Canada) at 35 and 25 cycles for IL-15 and actin respectively. The number of cycles for PCR was selected based on the linearity of the PCR product (data not shown). The cDNA concentrations were normalized to yield

equivalent actin PCR products to allow for comparison of IL-15 mRNAs. The cytokine-specific primer pairs, having sequences of IL-15A: (5'-ATGAGAATTTCGAAACCACATTTG-3'), and IL-15B: (5'-CCATTAGAAGACAAACTGTTCTTTGC-3'), β-actin A (5'CCTTCCTGGGCATGGAGTCCT-3') and β-actin B (5' GGAGCAATGATCTTGATCTTC-3'), were used. An aliquot of PCR product (20 µl) was electrophoresed on a 1 % agarose gel and visualized by ethidium-bromide staining. The specificity of the PCR products for IL-15 was confirmed by its predicted size on agarose gels and also by Southern blot analysis.

Southern Analysis

Amplified DNA was blotted on a positively charged nylon membrane (Boehringer, Mannheim) overnight at ambient temperature in a 10x saline sodium citrate buffer (ssc) [3 M NaCl, 300 mM sodium citrate, pH 7.0] and immobilized by UV cross-linking. The membrane was then prehybridized and hybridized at 58°C using rapid-hyb buffer (Amersham, Life Sciences) for 4 h and overnight, respectively. The synthesized PCR products were then probed with a labeled $P\text{-}^{32}$ IL-15 probe complementary to sequences recognized by the PCR amplification primers: (5'-ATGTCTTCATTTTGGGCTGTTTCAGTGCAG-3'). Amplified actin cDNA was detected using $P\text{-}^{32}$labeled human actin cDNA excised by EcoRI restriction enzyme from the actin containing 1.1-Kb plasmid (Bluescript SK-, ATCC). After hybridization, the blots were washed (15 min per wash) with solutions comprising 0.1% SDS: twice at ambient temperature with 2x ssc and once with 0.1x ssc at 37ºC, followed by 0.1x ssc at 58ºC. Probed products were quantified using a phosphoimager screen (Molecular Dynamics).

Statistical Analysis

Results are presented as mean ± SE. Statistical significance was determined using a student's *t*-test and $P<0.05$ was considered statistically significant.

RESULTS

Enhancement of NK activity by different viruses and its inhibition by anti-IL15 antibody

We first determined the NK cytotoxic activity of PBMC following their infection with different viruses as described in Materials and Methods. All viruses used (Influenza virus, HSV-1, EBV, Reovirus, VSV, Sendai virus, and RSV) were able to significantly enhance the NK activity of PBMC following 20 h of infection as compared to the mock-infected PBMC (Fig. 1). Infection with the influenza virus induced a 38 % increase in NK cytotoxicity as compared to 7% cytocidal activity of the mock-infected PBMC; these results correspond to more than 5.5-fold increase in cytocidal activity. Also, VSV had an activity of 43%, the highest increase in NK activity among the different viruses. RSV (15%) and EBV (20%) induced a lower increase in NK cytotoxicity. In order to determine the role of IL-15 in the observed induction of NK cytotoxicity, a mAb to IL-15 was used. It was thus found that this antibody inhibited the increase in NK activity following infection with the different viruses. This increase was statistically significant with a p value of $\leq$ 0.01 and was seen with all the viruses to a varying degree. Hence, the role of IL-15 in mediating the induction of NK activity following infection with Influenza virus, HSV-1, EBV, Reovirus, VSV, Sendai virus, and RSV was shown.

Specificity of NK induction by viruses via IL-15

The above observations pointed to a role for IL-15 in the upregulation of natural killer cytotoxicity by these different viruses. If this was true, the presence of bioactive IL-15 secreted in the supernatants of infected cells

should be detected. In fact, treatment of PBMC for 24 h with the supernatants of infected PBMC obtained 20 h P.I showed that all supernatants were able to significantly enhance NK activity as measured by percent cytotoxicity in comparison to the mock-treated PBMC (Fig.2). To document the specificity of NK induction by the supernatants of infected PBMC cultures via IL-15, a mAb to IL-15 was used. The results show that, when added to the supernatants, anti-IL15 antibody inhibited the induced increase in NK activity. The decrease in NK activity was statistically significant and observed with all viruses ($p \leq 0.01$). A more drastic decrease was seen with the supernatants of EBV and RSV (i. e. from 18% to 2% and from 24% to 3%, respectively), suggesting that in the case of these two viruses NK cytotoxicity was solely induced by IL-15. This data provides strong evidence for the presence of IL-15 in the supernatants of infected PBMC and its role in the induction of NK cytotoxicity.

Furthermore, the specific role of IL-15 in the NK induction by the supernatants of infected PBMC cultures by the different viruses was supported by the results of experiments using mAbs with specificity to other relevant cytokines such as IL-12, IL-2, TNF-α, and IFN-γ (Fig. 3). These results clearly showed a significant decrease in NK activity following the use of anti-IL15 antibody only and not with mAbs to other cytokines. This was demonstrated in each individual experiment with single viruses (Fig. 3, a-c). However, there was a slight non-significant decrease in NK activity with anti-IL-12 for the VSV and Sendai viruses. This decrease in cytocidal activity suggests a possible synergistic effect between IL-12 and IL-15 in the induction of NK activity. However, the NK activity decreased significantly only with the anti-IL-15 mAb. Taken together, these results clearly indicated that IL-15 was the cytokine specifically responsible for the upregulation of NK activity following infection of PBMC with different viruses.

Assessment of IL-15's bioactivity in the supernatants of infected PBMC

After ascertaining the presence of IL-15 in the supernatants of infected PBMC using a specific mAb, the following step was to test the bioactivity of the secreted protein. Attempts to detect IL-15 in the supernatants of infected PBMC by ELISA were unsuccessful (data not shown). In fact, the detection limits of most commercially available ELISA kits are probably well above the actual bioactive protein concentrations present in the supernatants of infected PBMC. Contrary to other cytokines, the concentration of IL-15 is not directly correlated with the cellular level of IL-15 mRNA expression (16, 17). However, we were able to detect bioactive IL-15 levels in the supernatants of infected PBMC 20 h post-infection using the CTLL-2 proliferation assay as described in Materials and Methods (Fig. 4). In fact, all supernatants obtained from virus-infected PBMC cultures supported the growth of the CTLL cell-line as illustrated by the significant increase in thymidine incorporation (cpm) (treatment of supernatants with a mAb to IL-2 ascertained that the proliferation of CTLL-2 was not due to IL-2). Interestingly, both the Influenza virus and VSV had the highest increase correlating with their highest induction of NK activity, whereas EBV and RSV supernatants showed lower values which correlated with their lower NK-inducing activity (Fig. 1).

Kinetics of IL-15 mRNA expression

In order to determine the levels of IL-15 mRNA expression at different time intervals (i. e. 2, 4, 8, 12 and 20 h) post-infection (P. I) of PBMC, cells were lysed and mRNA levels assessed by semi-quantitative RT-PCR (Fig. 6). IL-15 mRNA levels for most viruses peaked at 8 h P. I, with some variation among the different viruses: Influenza virus, HSV-1, EBV, and Sendai virus peaked at 8 h P. I, whereas Reovirus and RSV had little increase between 8 and 12 h P. I. (Fig. 5a, b), VSV peaked at 4 h P. I, probably accounting for the

higher increase observed in NK induction (Fig. 1). However, there were differences with respect to the correlation between the increase in mRNA levels and protein expression levels. For instance, EBV mRNA expression peaked at 8 h P.I, with a 40- fold increase in mRNA levels. In comparison, influenza IL-15 mRNA level also peaked at 8 h P.I with a 28-fold increase. For both viruses, the bioactive protein levels (Fig. 4), as well as the NK activity (Fig. 1) did not correlate with the observed mRNA levels. Compared to EBV, Influenza virus infection of PBMC was associated with a higher concentration of IL-15 protein and a much higher NK activity, thus pointing to the possibility of a differential regulation at the translational and post-translational levels of IL-15 expression. This is also true if one compares other viruses such as HSV-1 and influenza, which despite different expression levels of mRNA following infection (10- and 28-fold increase, respectively), induced NK activity of comparable strength and expressed similar levels of the bioactive protein (Fig. 1, and 4).

DISCUSSION

NK cells represent a distinct lineage of lymphocytes that plays an important role in the host's innate defense mechanisms aimed at the elimination of a variety of tumor, bone marrow transplanted, as well as virus-infected target cells (39, 41, 42). The cytocidal activity of NK cells has been shown to be enhanced by cytokines such as α and β IFNs produced by infected cells that upregulate killing mechanisms, and IL-12, which promotes IFN-γ secretion (19). The role of NK cells in early antiviral immune defense mechanisms seems to be crucial as evidenced by studies of several types of viral infections, particularly those due to Herpes viruses (39, 18). Numerous studies have demonstrated that NK cells can selectively lyse virus-infected target cells while sparing uninfected cells (20, 21). The expression of viral antigens or other surface structures by infected cells appears to render them more sensitive to NK cytolysis (20-22). Mammalian cells infected by many different viruses such as herpes, vaccinia, measles, mumps, and influenza viruses can be lysed *in vitro* by NK effectors (20). Furthermore, several studies have shown that humans and mice with a relatively low NK activity are more susceptible to herpesvirus infections such as herpes simplex, cytomegalovirus and EBV (20, 21, 23, 40, 50, 51). Characterization of the virus-induced endogenous mechanisms regulating NK cell responses and functions has been limited to the IFN α/β-mediated activation of NK cell cytotoxicity. Recent work has shown that some, but not all viral infections induce IL-12, the expression of which results in IFN-γ production by NK cells, which in turn contributes to an antiviral state (18). However, a newly identified cytokine, IL-15, with biologic activities similar to IL-2, including the ability to upregulate NK cytolytic mechanisms (5), has been shown to be induced following infection with two related human lymphotropic herpesviruses, HHV-6 and HHV-7 (7, 35). Furthermore, Elloso et al. (1996)

reported that neutralization of endogenous IL-15 in PBMC from HIV-infected patients, in which dysfunction of both NK and ADCC activity is well documented (43, 44), resulted in a reduction in IFN-γ production *in vitro* (25). A previous report had shown that IL-15 and IL-12 had the ability to enhance cell mediated immunity of HIV-infected PBMC (24), and recent work from our laboratory has shown that adding rIL-15 to PBMC culture from HIV-infected individuals could upregulate NK and ADCC activity *in vitro* (52). Taken together, these data suggest that IL-15 plays an important role in the generation of antiviral state mechanisms following viral infection.

Other recent studies suggested that IL-15 may be playing an important role in the induction of the local T cell response to human intracellular pathogens. This is illustrated by the example of Mycobacterium leprae (*M. leprae*), whereby resistance to the disease and a Th1 cytokine pattern of cell-mediated immunity correlated with IL-15 mRNA expression (26). PBMC were found to secrete IL-15 following infection and rIL-15 in combination with *M.leprae* induced the expansion of a population of CD3+CD56+ T cells (26). [Work from our laboratory showed that incubation of rIL-15 with PBMC from normal individuals for 21 days induced the proliferation of CD3+CD56+ T lymphocytes significantly (45)]. In addition, Hunter et al. (1997) showed that IL-15 messages were also present in tissues of SCID mice infected with *T. gondii* (27). Taken together, these studies point to an important role for IL-15 in viral, bacterial and parasitic infections.

In order to gain further insight about the role of IL-15 in the activation of the non-specific cellular immune response to viral infection, we studied the induction of IL-15 following exposure of human PBMC to several viruses belonging to different families; namely the Influenza virus, Herpes simplex-1, Epstein-Barr virus, Reovirus, Vesicular Stomatitis virus, Sendai virus, and Respiratory Syncitial virus, and its role in the enhancement of the natural

killer cytotoxic activity of infected PBMC. Our data clearly shows that all the viruses studied induced the expression of IL-15, both at the mRNA and protein levels, and that they significantly enhanced the NK activity of PBMC as compared to the mock-infected cells. This increase in NK cytotoxicity was shown to be abrogated by the use of a mAb to IL-15. Furthermore, when the supernatants from virus-treated PBMC cultures were added to normal PBMC, the NK activity of the latter cells was upregulated. These data thus show that NK activity is upregulated following viral infection via IL-15 induction. Furthermore, the presented data clearly shows that IL-15 was specifically responsible for the NK induction of normal PBMC following treatment with supernatants of infected PBMC. Inhibition of NK activity was only seen with anti-IL15 and not with mAbs specific to other cytokines such as IL-12, IL-2, IFN-γ, or TNF-α. This suggests that IL-15 could be secreted earlier than the other cytokines following PBMC infection. This is supported by the fact that monocytes are the primary source of IL-15 production in response to intracellular infection, as documented in several studies (8, 28-30). These findings indicate the importance of studying the role of IL-15 in infection and its potential role in the production of other cytokines.

It has been difficult to demonstrate IL-15 in the supernatants of the majority of the cells that express messages for this cytokine, despite its widespread expression in several human tissues (17). The results obtained in the presented study regarding the mRNA and protein expression levels of IL-15 illustrate the differential regulation of this cytokine. In fact, IL-15 mRNA levels were not translated into equivalent proportions of bioactive protein as assessed by the CTLL-2 bioassay. This could be explained in the light of the translational and post-translational (secretion) levels of regulation of IL-15 protein expression. Unlike other cytokines that belong to the same family, such as IL-2 which is regulated at the level of message transcription and stabilization, IL-15's synthesis and secretion appear to be controlled by the

presence of upstream AUGs in the 5'UTR region of the IL-15 messages (16, 17). It has been hypothesized that IL-15 is stored in a translationally inactive IL-15 mRNA form that can be readily translated in response to an infection through several mechanisms that are effective in the removal of the 5′ UTR blockade of transcription such as a splicing event or an internal initiation of translation (31, 46, 47). Interestingly, Bamford et al. (1996) showed by analysis of the IL-15 message from the HuT-102 T cell line, that there was a 6- to 10-fold more protein expressed in HuT-102 cells as compared to activated monocytes, correlating with the lack in HuT-102 cells of 8 of the 10 upstream AUGs normally present in the 5'UTR of the IL-15 message (16). Furthermore, IL-15's secretion was found to be controlled by natural signal peptides that apparently regulate the efficiency of release of soluble IL-15 in biologically relevant amounts (32, 33). Meazza et al. (1997) reported that substitution of the natural signal peptide encoded by IL-15 cDNA by another one from a secretory protein IgVκ chain (VκL) increased significantly the secretion of biologically active IL-15 (32). Hence, the results obtained in the present study are consistent with other findings, suggesting that IL-15's synthesis and secretion is controlled at multiple levels (translation and entry into the secretory pathway), in addition to transcription (16).

The study of innate immunity and its consideration as a separate entity underlines its importance in immune surveillance against infection. Moreover, unraveling the role of cytokines involved in the regulation of the early immune response to infections could lead to a better understanding of their potential role in pathogenesis. With respect to IL-15, the results presented confirm its role in the activation of NK cytocidal activity. In this context, it is noteworthy that a recent study by Carson, et al (1997), documented the ability of IL-15 to sustain NK cell survival and that NK cells expressed IL-15Rα mRNA (34). It was shown that picomolar amounts of IL-15 were sufficient, in the absence of serum or other growth factors, to sustain the survival of resting human NK

cells for up to 8 days. It is thus likely that IL-15 plays a unique role in the activation and maintenance of the host's innate cellular immune response to infection.

Acknowledgments

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FIGURES AND LEGENDS

Figure 1: *Induction of NK activity by different viruses and inhibition by a mAb to IL-15*

Freshly isolated PBMC ($1x10^6$ cells) were infected with optimal doses of different viruses (Influenza, HSV-1, EBV, Reo, VSV, Sendai, RSV), as described in Materials and Methods. Cells were incubated with the viruses for 2 h, and then resuspended in 1 ml RPMI-1640 with 10% FBS in the presence or absence of a mAb to IL-15 (10 μg/ml) for 20 h at 37°C in 5% CO_2. Cytolytic activity was measured by mixing infected PBMCs with K562 cells at an E:T ratio of 20:1, and their cytotoxic activity was assayed in a 16-hr ^{51}Cr release assay as described above. Data, expressed as percent cytotoxicity, represents mean ± SE with a p value ≤ 0.01 obtained from triplicate experimental values for one donor. These experiments were repeated with PBMC from four different donors, and similar results were obtained for each donor. Black bars represent infected cells treated with anti-IL-15 mAbs.

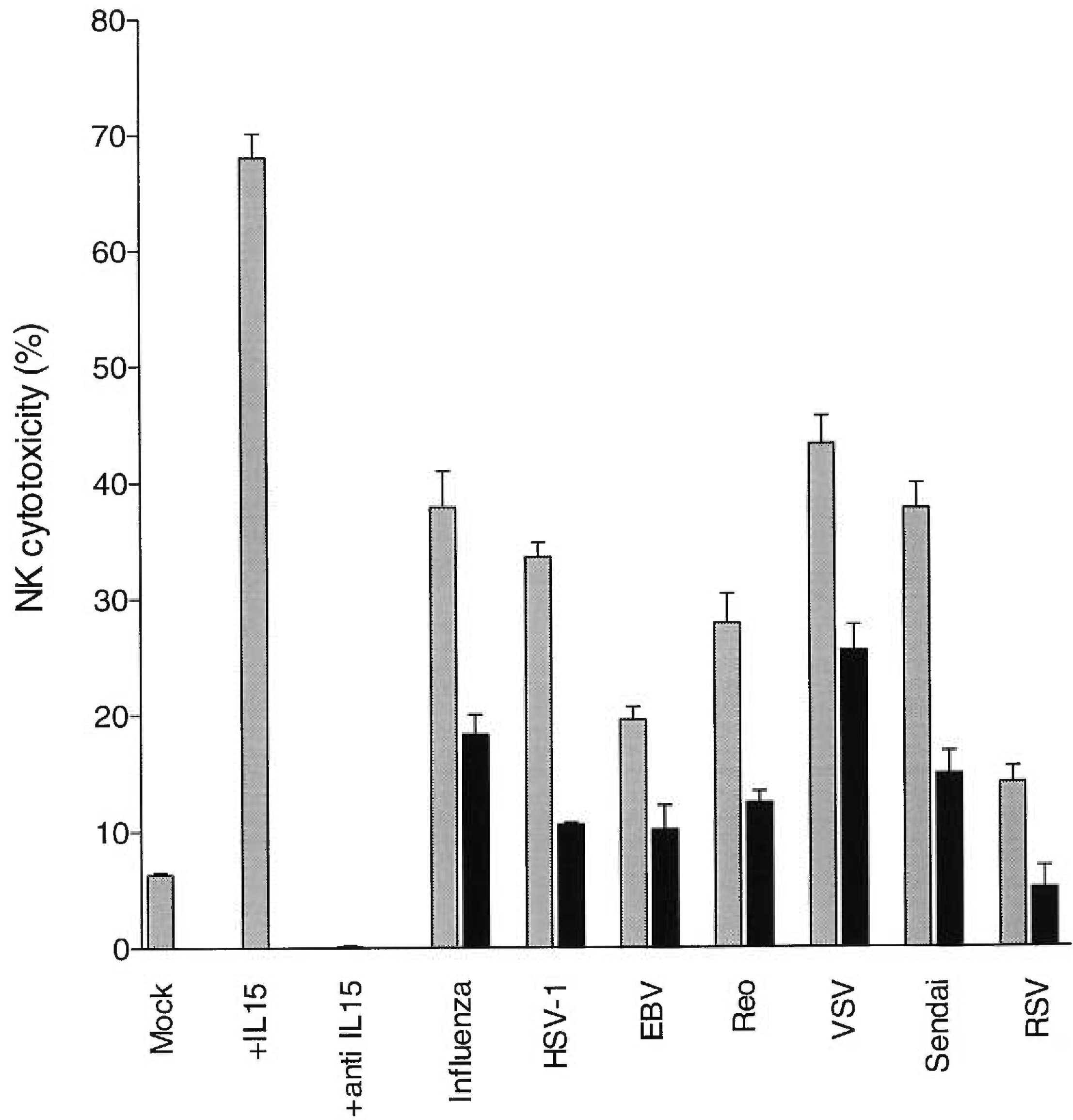

Figure 1: Induction of NK activity by different viruses and abrogation by a monoclonal antibody to IL-15

Figure 2: *Induction of NK cytotoxicity by supernatants from virus-infected PBMC cultures, and the effect of anti-IL15 on this activity*

PBMC obtained from healthy donors were assayed for their NK cytotoxicity after a 24 h-treatment with supernatant from infected cell cultures (at 25% of total volume) in the standard 16-hr ^{51}Cr release assay using K562 targets, in the presence or absence of a mAb to IL-15 (10 μg/ml). The supernatants used were collected 20 h post-treatment of freshly isolated PBMC with the different viruses and mock-supernatant, as described above. Data are expressed as percent cytotoxicity, representing mean ± SE with a p value ≤ 0.01 obtained from triplicate experimental values. Results obtained from other experiments carried out using PBMC from four different donors were similar. Black bars represent cytotoxic activity of infected PBMC treated with anti-IL15 mAbs.

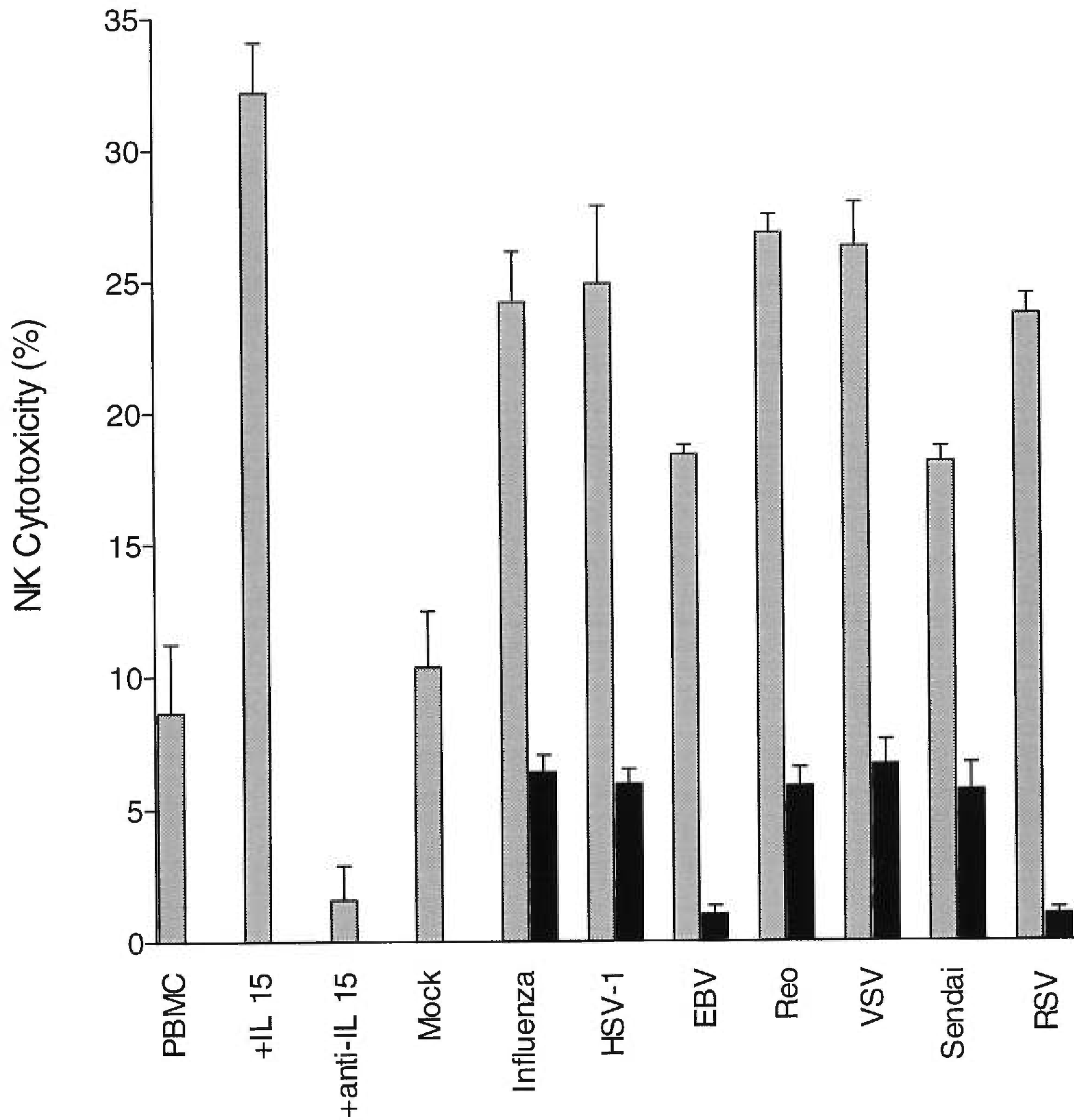

Figure 2: Induction of NK cytotoxicity by supernatants of virus-infected PBMC cultures, and the effect of anti-IL 15 on this activity

Figure 3: *Specific role of IL-15 in NK induction by different viruses*

Freshly isolated PBMC ($1x10^6$ cells), obtained from healthy donors, were infected with optimal doses of the different viruses or mock-infected for 2h at 37°C in 5% CO_2. Cells were then washed and resuspended in RPMI-1640 with 10% FBS for 20 h at 37°C in 5% CO_2. Culture supernatants were then collected and added to resting PBMC ($4x10^6$ cells/ml) at 25% of final volume in the presence of mAbs to IL-15, IL-2, IL-12, TNF-α, and IFN-γ (all antibodies were used at a concentration of 10 μg/ml). The lytic activity was measured in a standard 16-hr ^{51}Cr release assay using K562 targets at a 20:1 effector to target ratio as described in Materials and Methods. a) Reovirus, RSV; b) HSV-1, VSV; c) Sendai virus, Influenza virus. Values obtained represent mean ± SE of triplicate experimental values. Results obtained from other experiments done using PBMC from three different donors were similar. Stars show that addition of anti-IL-15 significantly reduced NK activity induced with the viral supernatants with a p value ≤0.02.

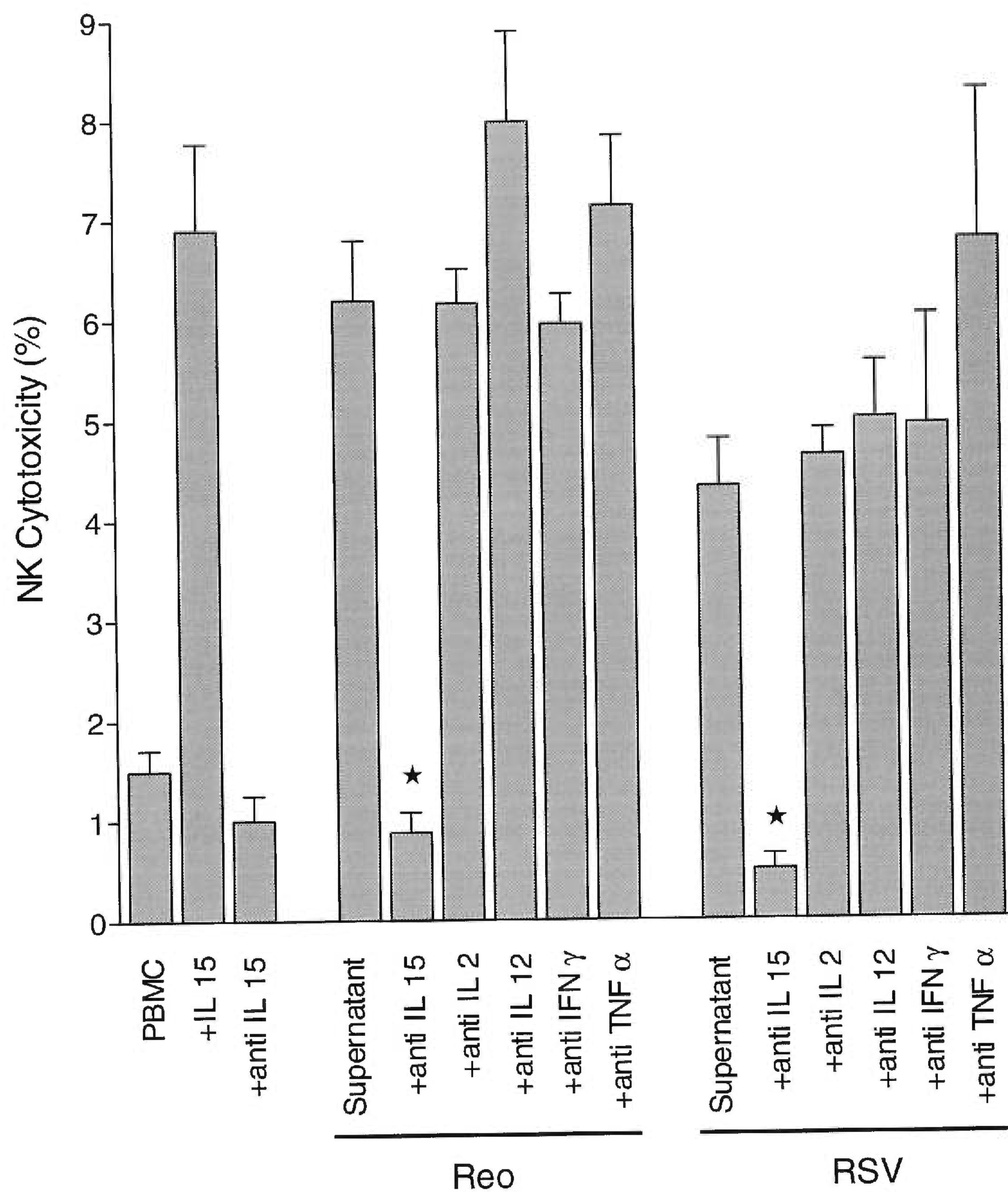

Figure 3,a: Specific role of IL-15 in the NK induction by different viruses

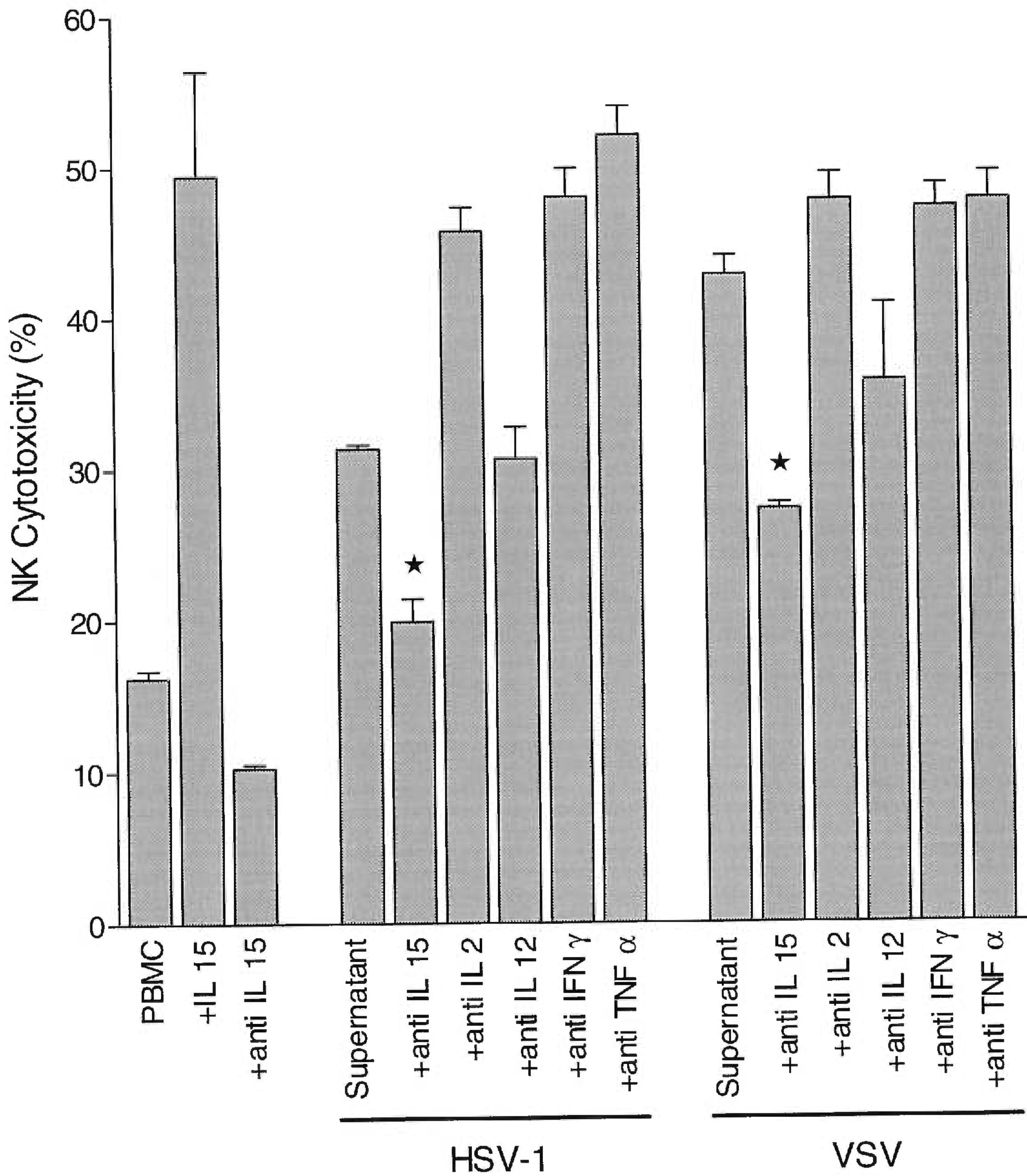

Figure 3,b: Specific role of IL-15 in the NK induction by different viruses

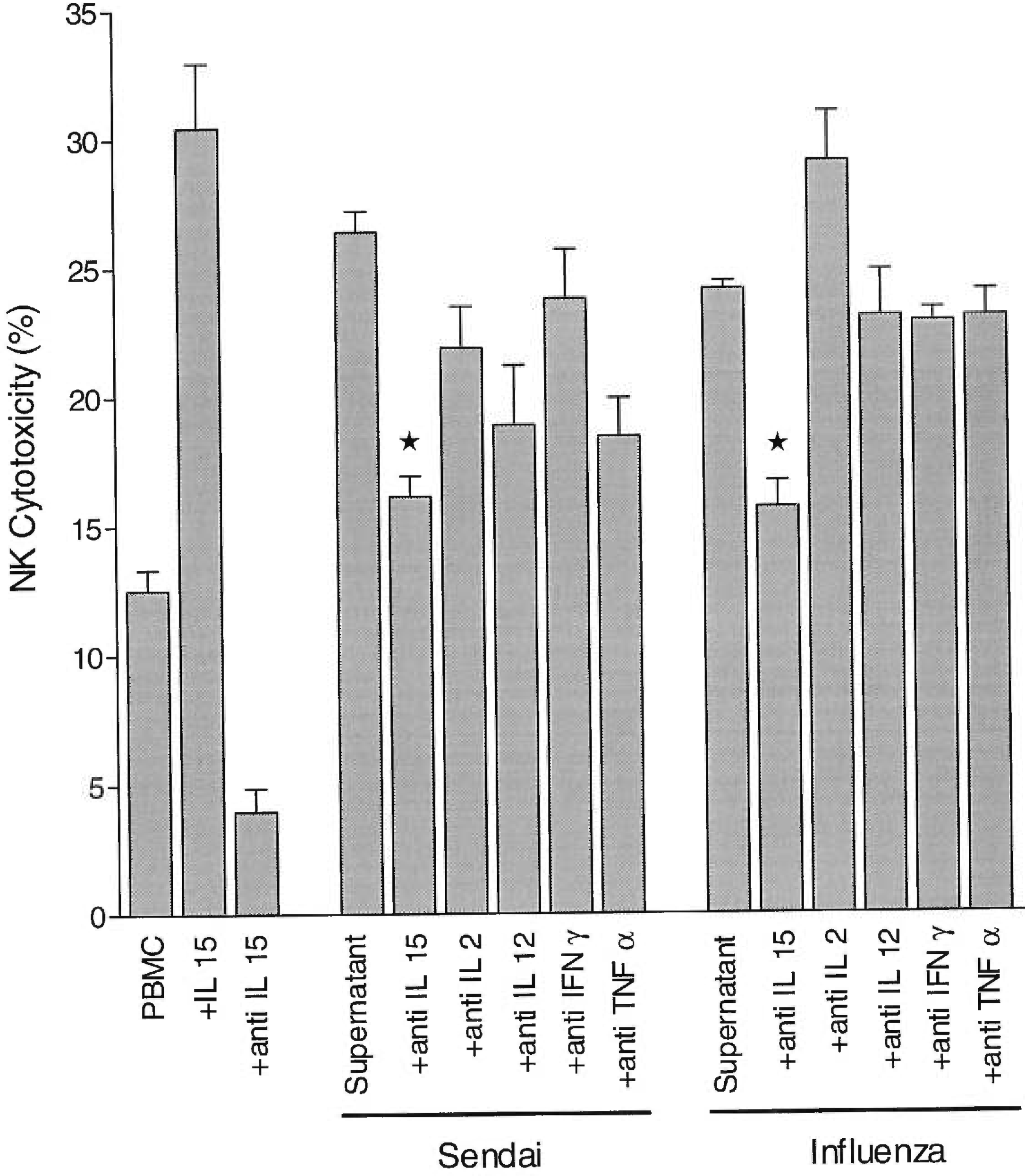

Figure 3,c: Specific role of IL-15 in the NK induction by different viruses

Figure 4: *IL-15 bioactivity in the supernatants of infected PBMC cultures 20 h post-infection as measured by CTLL-2 assay*

Fresh PBMC were obtained from healthy donors and infected with different viruses for 20 h at 37°C in 5% CO_2. Their supernatants were added (100 μl) to CTLL-2 cells ($5x10^3$ cells/100 μl) into 96-well round bottom plate. After a 66 h incubation, cells were pulsed with thymidine for 6 h and bioactive IL-15 present in the virus-infected and mock-infected PBMC culture supernatants was assessed by its ability to sustain the proliferation of CTLL-2 cells. Supernatants had been previously treated with anti-IL2 to ensure that proliferation was solely due to IL-15. Results are expressed as cpm thymidine incorporation, and each value represents the mean of assays performed in duplicates. The results are representative of two separate experiments.

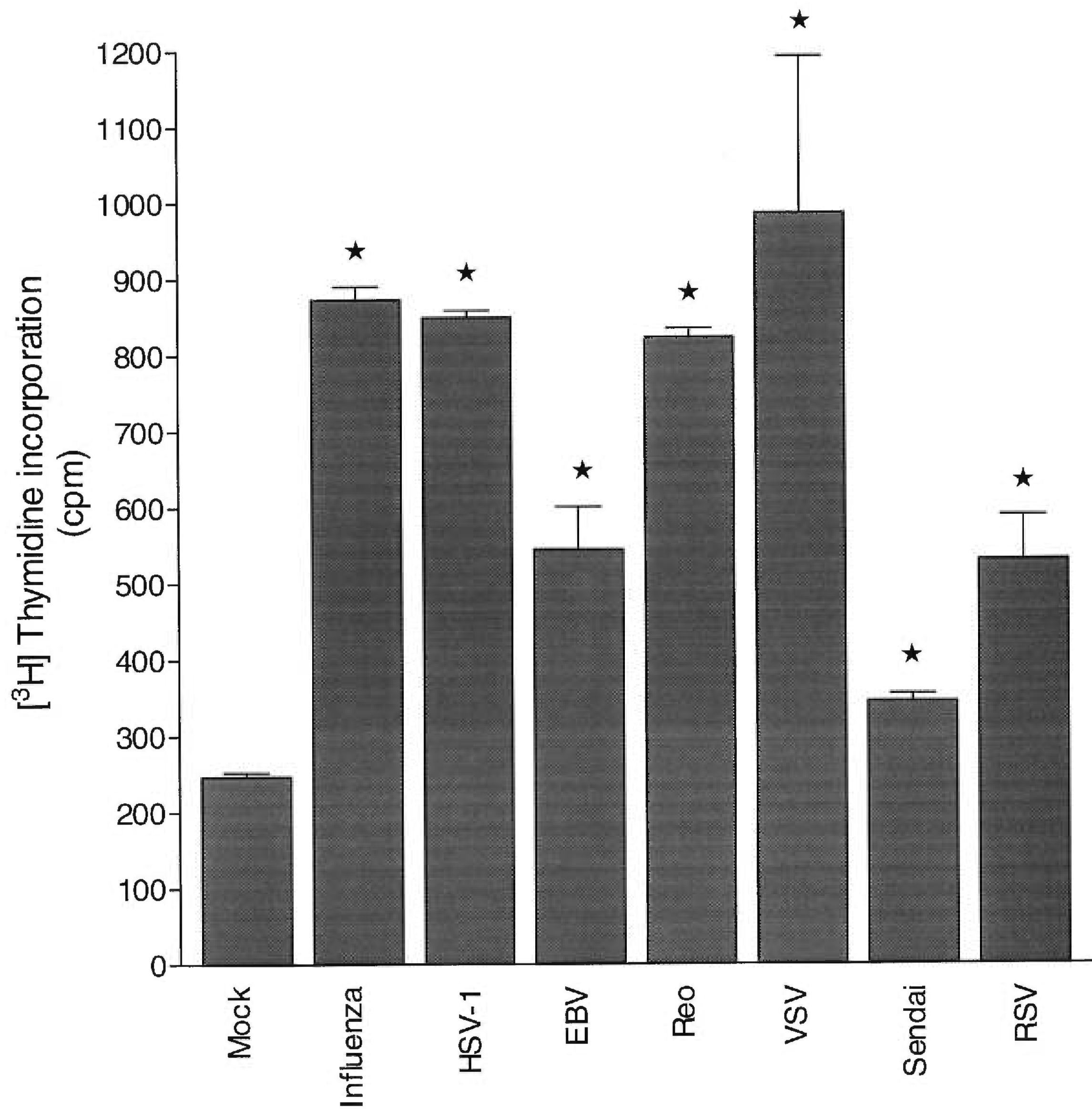

Figure 4: IL-15 bioactivity in the supernatants of infected PBMC cultures 20 h post-infection as measured by CTLL-2 assay

Figure 5: *Kinetics of IL-15 mRNA levels in infected PBMC as determined by RT-PCR*

PBMC ($1x10^6$ cells) were treated with different viruses for 2 h at 37°C, washed and resuspended in 1 ml RPMI-1640 supplemented with 10% FBS. At various time intervals (2, 4, 8, 12 and 20 h) post-treatment, cells were counted, and $5x10^5$ cells were lysed and assayed for IL-15 mRNA levels. Total RNA was extracted, reverse-transcribed, and amplified using RT-PCR. Bands were detected following blotting on a nylon membrane as described in Materials and Methods. A: The panels represent bands obtained in mock and virus-infected cells for IL-15 and actin at the different time intervals post-infection. B: Curves illustrating fold increase in IL-15 mRNA levels as expressed for different virus infected cells.........

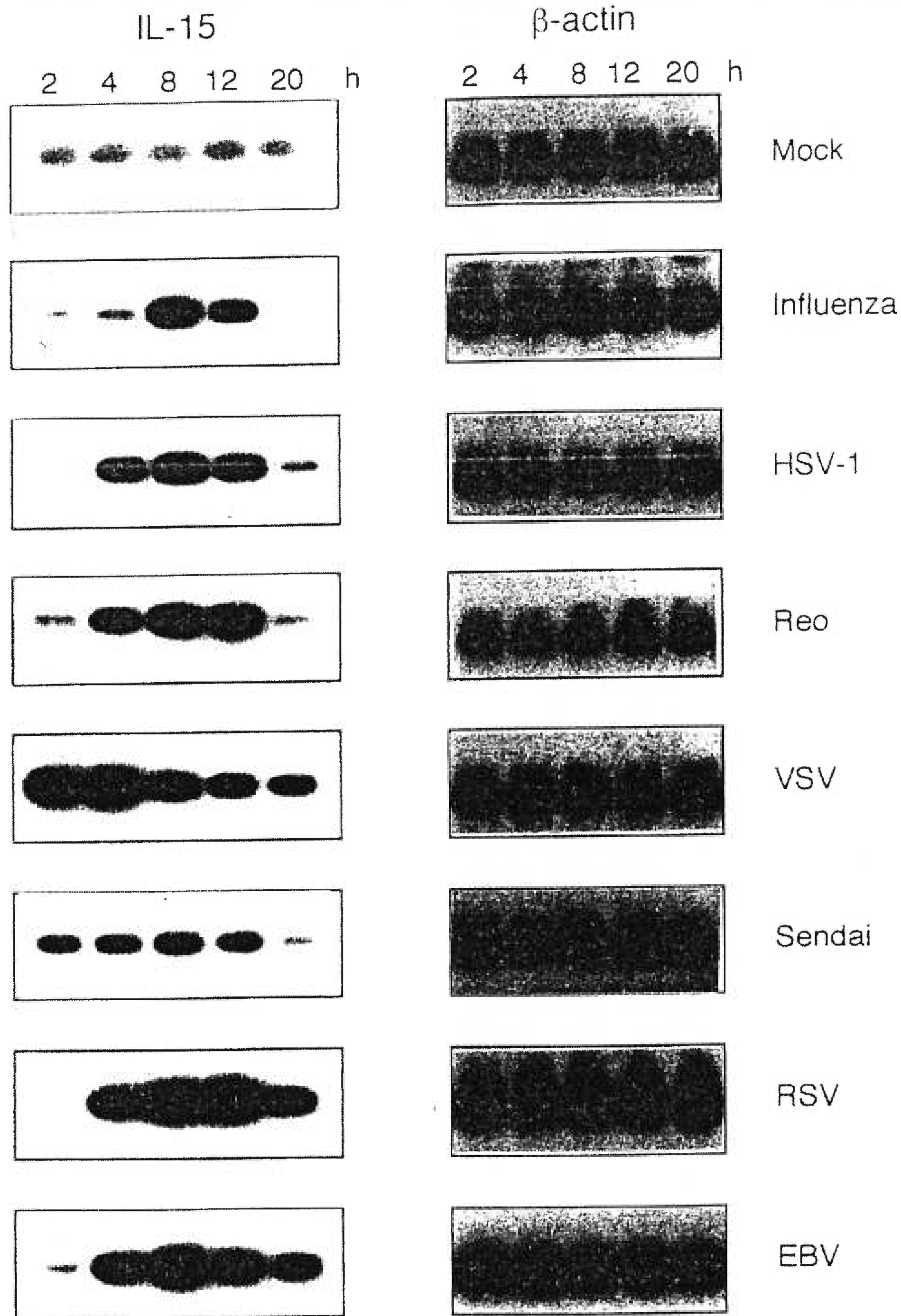

Figure 5, a: Kinetics of IL-15 mRNA levels in infected PBMC as determined by RT-PCR

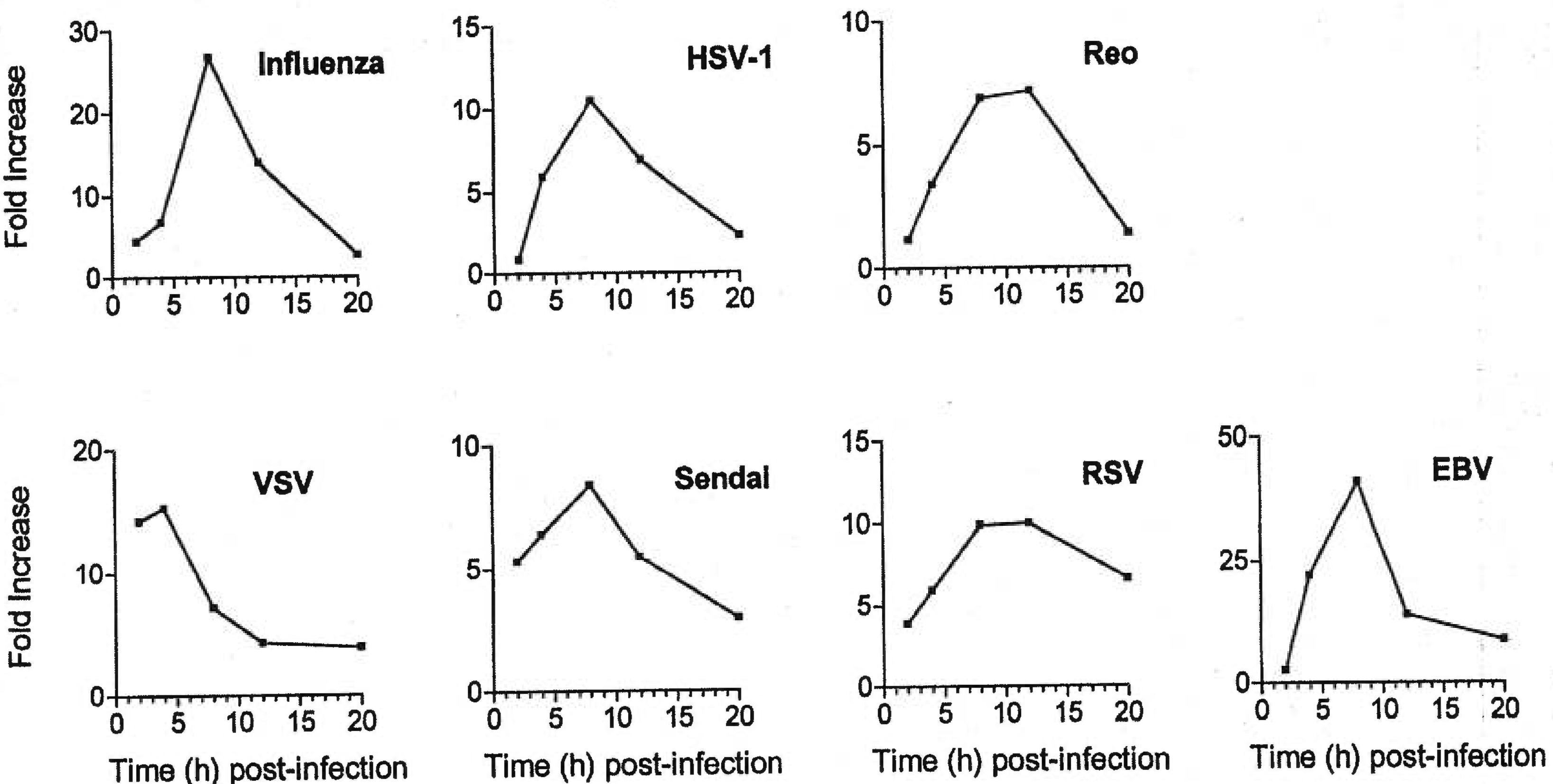

Figure 5, b: Kinetics of IL-15 mRNA levels in infected PBMC as determined by RT-PCR