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HUMAN NATURAL INTERFERON- α PRODUCING CELLS

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Abstract—Interferons (IFNs) are critical components of the host immune system, serving as antiviral agents, immunomodulators and inhibitors of cell growth. Among peripheral blood mononuclear cells, the primary IFN- α -producing cell is a light density, HLA-DR⁺ cell negative for cell surface markers typical for T cells, B cells, monocytes, natural-killer or progenitor cells and has been tentatively termed the ‘natural IFN-producing cell’ or NIPC. Although present in very low frequency (approximately 1:1000 among peripheral blood mononuclear cells), the NIPC are very potent, with an individual cell able to produce 1-2 IU of IFN. In this review, the characteristics, phenotype, regulation and relationship of NIPC to human disease are discussed.

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1. INTRODUCTION

Host defense against infectious disease is complex, involving interactions between components of both the innate (or natural) and the adaptive immune systems. Interferons (IFNs), which are produced in response to a variety of stimuli, including viral or microbial infections, are some of the central players in these host resistance mechanisms. The interferons were first described more than 30 years ago for their ability to protect uninfected cells from viral challenge. In the classic

Abbreviations—AC, accessory cell; AIDS, acquired immune deficiency syndrome; CMV, cytomegalovirus; gD, glycoprotein D; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IFN, interferon; MHC, major histocompatibility complex; NIPC, natural interferon-producing cell; NK, natural killer; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; TGEV, transmissible gastroenteritis virus.

experiments of Isaacs and Lindenmann, a soluble factor produced in response to influenza virus infection of chick chorioallantoic membrane was able to confer protection or 'interfere' with subsequent viral infection of previously noninfected cells (Isaacs and Lindenmann, 1957; Lindenmann, 1981). These interfering substances, now known as IFNs, were subsequently found not only to be able to induce an antiviral state in uninfected cells, but are also potent antimicrobial, antitumor and immunomodulating agents and have the ability to inhibit cell growth.

Although officially defined as substances that 'exert virus nonspecific antiviral activity through cellular metabolic processes involving synthesis of both RNA and protein' (Stewart, 1980), it is recognized that interferons are a heterogeneous family of proteins with varying molecular weights and biological functions (Pestka *et al.*, 1987; Pestka, 1986; Rubinstein *et al.*, 1978, 1979, 1981). The major subgroupings of IFNs include IFN- α (or leukocyte IFN), IFN- β (or fibroblast IFN), which together make up 'Type I IFN', and IFN- γ , also known as 'Type II' or 'immune' IFN. IFN- α and IFN- β are molecularly related species that share a common high-affinity cell-surface receptor, whereas IFN- γ is unrelated to either IFN- α or IFN- β and binds to a distinct cell-surface receptor (Pestka *et al.*, 1987; Langer and Pestka, 1988; Merlin *et al.*, 1985; Aguet *et al.*, 1988; Uze *et al.*, 1990). In humans, the genes for IFN- α and IFN- β are located on the short arm of chromosome 9 (Shows *et al.*, 1982). The human IFN- α supergene family consists of 14 nonallelic genes plus four pseudogenes. Also included within the IFN- α gene family is the single gene for IFN- ω , which shares approximately 70% homology with other human IFN- α genes and six IFN- ω pseudogenes. In humans and mice, IFN- β is coded for by a single gene exhibiting approximately 50% homology with IFN- α genes, whereas other species, including cows, sheep and pigs, possess multiple IFN- β genes (reviewed in Dron and Tovey, 1992). The genes of the IFN- α/β family share the property that they lack intervening sequences (Weissmann and Weber, 1986).

1.1. INTERFERON PRODUCTION IN *IN VITRO* CULTURE

In older IFN nomenclature, the Type I IFNs were designated 'leukocyte' or 'fibroblast' IFNs, reflecting a relative tissue specificity for the production of these subtypes. These two IFN subtypes, which are now designated as IFN- α and - β , respectively, are now known to be produced by a number of different cell types, with the most efficient producers being among the hematopoietic and embryonic cells. Although it is recognized that many cell types have the potential to produce IFN- α or - β , much of the attention in the literature has focused on the production of IFN by human peripheral blood mononuclear cells (PBMC) following *in vitro* stimulation with viral or other inducers. This focus on production of IFN by leukocytes probably reflects both the relatively easy accessibility of human blood as well as the interest in studying interactions between the cells comprising the peripheral blood as a microcosm of the immune response that occurs at the organismal level.

There has been much conflicting data in the literature regarding the cellular origin of IFN- α/β following stimulation with a number of different viral, bacterial, mycoplasmal and tumor cell agents, or natural or synthetic components, including viral glycoproteins and synthetic double-stranded RNA, such as polyI:polyC (Torrence and De Clercq, 1981). In these different systems, IFN production has been attributed to either B cells (Weigent *et al.*, 1981; Wiranowska-Stewart and Stewart, 1981; Capobianchi *et al.*, 1988b), monocytes (Stanwick *et al.*, 1982; Saksela *et al.*, 1984), natural killer (NK) cells (Timonen *et al.*, 1980; Djeu *et al.*, 1982; Merrill *et al.*, 1984; Kurane and Ennis, 1987), dendritic cells (Perussia *et al.*, 1985), or to a poorly defined population of 'null' cells (Peter *et al.*, 1980; Rönnblom *et al.*, 1983a,b; Kurane and Ennis, 1987). The reasons for these different attributions may reflect cellular purification techniques, methods of IFN induction (e.g. culture duration, cell density), as well as the nature of the IFN-stimulating agents themselves. Most recently, attention has focused on a population of cells within the peripheral blood, which is nonphagocytic, major histocompatibility complex (MHC) Class II positive, but negative for a variety of markers characteristic of T cells, B cells, NK cells or monocytes (Abb *et al.*, 1983; Fitzgerald *et al.*, 1984; Perussia *et al.*, 1985; Bandyopadhyay *et al.*, 1986; Fitzgerald-Bocarsly *et al.*, 1988; Feldman and Fitzgerald-Bocarsly, 1990). These cells, which may represent a unique cell type, have tentatively been termed 'natural interferon-producing cells' or NIPC (Rönnblom *et al.*, 1983b).

The majority of work on the identity of IFN-producing cells has focused on two viruses, namely Sendai virus and Herpes simplex virus (HSV). The reasons for these foci of activity are that Sendai is the prototypical inducer of IFN in PBMCs (Cantell *et al.*, 1981) and because HSV is a major human pathogen. Our interest in HSV comes from our studies on the role of natural resistance mechanisms against HSV and the finding that IFN- α is produced during the course of 14-hr NK assays against HSV-infected fibroblasts (Fitzgerald *et al.*, 1982). The production of predominantly IFN- α in response to HSV-infected fibroblasts, as well as to UV-inactivated HSV, was confirmed by the acid stability of the IFN, cross-reactivity on bovine cells and its ability to be neutralized by antibodies to IFN- α but not by antibodies against IFN- β or - γ .

1.2. INTERFERON PRODUCTION IN RESPONSE TO SENDAI VIRUS

Sendai virus is an enveloped, negative-stranded RNA virus of the *Paramyxovirus* genus of the family Paramyxoviridae (Kingsbury, 1990). This murine virus is closely related at the molecular level to Newcastle disease virus, whose natural host is the chicken, the mumps virus that infects humans, as well as to the human parainfluenza virus types 1-4. Sendai virus was shown early on to be a potent stimulator of IFN from leukocytes and is used as the stimulator in the commercial production of human leukocyte IFN (Cantell *et al.*, 1981). Sendai virus is also able to induce the production of IFN- α by the human lymphoblastoid cell line Namalwa. Among PBMCs, the monocyte has been most often associated with production of IFN in response to Sendai virus. Plastic adherent cells were shown by Saksela *et al.* (1984) to be the primary producers of IFN in response to Sendai virus. Moreover, cells bearing the CD14 monocyte-associated cell surface marker were found to be the predominant cells capable of producing IFN in response to free Sendai virus (Sandberg *et al.*, 1990, 1991b). This same group demonstrated that a cell, with a frequency of approximately 1:50 among PBMC and which had monocytic morphology, was positive for IFN- α 2 mRNA as assessed by *in situ* hybridization following induction of PBMC with Sendai virus (Gobl *et al.*, 1988). Our studies, however, have indicated that in addition to a CD14⁺ cell, a nonadherent, nonmonocytic cell is also capable of producing IFN in response to Sendai virus (M. Milone and P. Fitzgerald-Bocarsly, manuscript in preparation). Moreover, cell-associated Sendai virus that was fixed to the cell surface of WISH epithelial cells failed to induce IFN production in monocytes, but rather stimulated a smaller population of cells, presumably the NIPC (Sandberg *et al.*, 1990).

1.3. IFN PRODUCTION IN RESPONSE TO HERPES SIMPLEX VIRUS

HSVs have been known for some time to be potent stimulators of IFN production in PBMC. Neutralization studies have demonstrated that most or all of the IFN induced by HSV type 1 in short-term cultures could be neutralized by antisera to human leukocyte IFN (Green *et al.*, 1981; Fitzgerald *et al.*, 1982; Bishop *et al.*, 1983). However, following long-term culture with HSV (3 days), HSV seropositive individuals also produce IFN- γ (Green *et al.*, 1981), which presumably comes from sensitized T cells and possibly NK cells. Live HSV, UV-inactivated HSV, live HSV-infected fibroblasts and fixed HSV-infected cells have all been shown to induce IFN- α in PBMC populations. Although the cells responsible for IFN- α production in response to HSV have occasionally been reported to be B cells (Capobianchi *et al.*, 1988b), the most thorough studies have indicated that the predominant IFN-producing cells are negative for cell surface markers typical of B cells or T cells (Peter *et al.*, 1980; Abb *et al.*, 1983; Rönnblom *et al.*, 1983a,b; Fitzgerald *et al.*, 1984; Perussia *et al.*, 1985; Bandyopadhyay *et al.*, 1986; Fitzgerald-Bocarsly *et al.*, 1988; Feldman and Fitzgerald-Bocarsly, 1990). Moreover, although they share some overlap with NK cells when separated on Percoll density gradients (Djeu *et al.*, 1982; Fitzgerald-Bocarsly *et al.*, 1988), the peak IFN activity is found in Percoll gradient fractions slightly less dense than for the NK cells (Fitzgerald-Bocarsly *et al.*, 1988). High levels of IFN generation can be observed among cells rigorously depleted of esterase-positive cells (Fitzgerald-Bocarsly *et al.*, 1988), indicating that at least a majority of IFN production in response to HSV cannot be attributed to monocytes. Although lacking a variety of other cell markers, IFN- α -producing cells have been found to express MHC Class I (Fitzgerald-Bocarsly *et al.*, 1988) and Class II (Abb *et al.*, 1983; Perussia *et al.*, 1985;

Fitzgerald-Bocarsly *et al.*, 1988) antigens, such that depletion of either Class I-positive or HLA-DR⁺ cells eliminates IFN production in response to HSV. In addition to MHC determinant expression, elegant flow cytometry experiments of Sandberg *et al.* have indicated that these IFN-producing cells express low levels of CD4 (Sandberg *et al.*, 1990) and approximately 50% express the thrombospondin receptor, CD36 (Sandberg *et al.*, 1989). The presence of the D44 antigen, which is also present on immature hematologic cells and on a subpopulation of T cells (Bernard *et al.*, 1984), has also been reported on the NIPC (Lebon *et al.*, 1982; Chehimi *et al.*, 1989). Further identification of the cells responsible for production of IFN- α in response to HSV have been hampered by their failure to express any known unique cell surface determinants and their paucity in peripheral blood. Whether these cells truly represent a unique lineage or whether they represent a subset of some known lineage remains to be determined.

1.4. IFN PRODUCTION IN RESPONSE TO OTHER INDUCERS

Although Sendai and HSV represent the best studied of the viruses for induction of IFN- α/β in PBMC, the IFN response to a number of other viruses, as well as mycoplasma, tumor cells and bacteria, have also been studied. In some of the earlier studies, IFN production was attributed to either B cells or B cell-enriched populations (Wiranowska-Stewart and Stewart, 1981). However, close examination of the experimental protocols reveals that these populations were actually depleted of macrophages (by adherence) and T cells (by E rosetting), yielding populations of cells that contained not only B cells, but null cells as well. In addition, although a number of studies attribute IFN production to monocytes, these studies typically utilize adherent cells, which may contain other populations of cells as well.

2. CHARACTERIZATION OF NATURAL IFN-PRODUCING CELLS

2.1. CELL SURFACE MARKERS ON NIPC

As described in Section 1.1, the population of NIPC was initially described as a null cell population, since it lacked many of the cell surface markers characteristic of mature T cells, B cells, NK cells and monocytes. The presence of MHC Class II antigens has suggested the possibility that NIPC can function as antigen-presenting cells to CD4⁺ T cells (Sandberg *et al.*, 1989). This hypothesis has not been tested, however, due to the inability to purify viable NIPC to homogeneity for testing as antigen-presenting cells.

The relationship of NIPC to monocytes has been further studied. Cells rigorously depleted of esterase-positive monocytes by adherence to plastic, nylon wool and Sephadex G-10 were found to produce high levels of IFN- α in response to HSV (Fitzgerald-Bocarsly *et al.*, 1988). Moreover, the IFN-producing cells were not depleted by phagocytosis of carbonyl iron followed by magnetic separation (Fitzgerald-Bocarsly *et al.*, 1988; Feldman and Fitzgerald-Bocarsly, 1990) or by phagocytosis of latex particles (Sandberg *et al.*, 1990), a treatment that was shown to deplete Sendai-induced monocyte IFN production. These studies, however, were based on depletion rather than enrichment of monocytes, and, therefore, did not adequately address the issue of a monocytic subpopulation capable of generating IFN. Along these lines, we have observed variable amounts of IFN production by plastic or serum-coated plastic adherent cells in response to HSV. However, plastic adherent cells are not uniformly monocytic, since other populations, including dendritic cells, can transiently adhere to plastic (Knight *et al.*, 1986; Rasanan *et al.*, 1988). To address the issue more directly, Sandberg *et al.* (1991b) have sorted unstimulated PBMC using a variety of markers found on cells in the myeloid lineage. Following selection by cell sorting, the cells were stimulated with glutaraldehyde-fixed HSV-WISH, then tested for IFN- α 2 mRNA by *in situ* hybridization. The NIPC were found to lack CD33, CD13, CD11b, CD14 and CD15 markers, arguing against a myeloid lineage for these cells. However, in recent studies, we have separated PBMC into CD14-positive and -negative fractions using immuno-magnetic beads and tested the ability of the positively selected cells to produce IFN (M. Milone and P. Fitzgerald-Bocarsly, manuscript in preparation). The CD14⁺ cells (which were up to 97% purified) produced 300 IU of IFN in response to HSV stimulation (vs 1000 IU for Sendai-induced cells) and contained up

to 0.05% HSV-responsive IFN-producing cells as measured by ELISpot assay. Thus, it appears that at least a fraction of the cells responding to HSV are CD14⁺. Whether these represent true monocytes or the co-expression of CD14 by another cell type remains to be determined. As expected, IFN-producing cells in response to Sendai virus were highly enriched in the CD14⁺ cells.

Although our studies using a panning technique indicated that positively selected CD4 cells did not generate IFN- α in response to HSV-1 (Siegal *et al.*, 1986; Fitzgerald-Bocarsly *et al.*, 1988), Sandberg *et al.* (1990) have identified low-density CD4 antigens on cells that have been pre-stimulated with HSV-WISH cells, followed by cell sorting and *in situ* hybridization for IFN- α mRNA. The presence of CD4 antigens on the NIPC is interesting in light of the observations of depressed IFN- α production in patients with the Acquired Immune Deficiency Syndrome (AIDS) (Lopez *et al.*, 1983; Siegal *et al.*, 1986; Rossol *et al.*, 1989) and the possibility that the NIPC themselves may be targets for infection with the human immunodeficiency virus (HIV) (see discussion of this in Section 6.1). Sandberg and colleagues (1989) have also demonstrated the presence of CD36, the thrombospondin receptor, on about half of the NIPC. This molecule is also found on monocytes, platelets, a small proportion of null cells and vascular endothelial cells. In addition, other studies have shown NIPC can be eliminated by treatment with anti-D44 mAb and complement (Lebon *et al.*, 1982; Chehimi *et al.*, 1989). D44 is an antigen that is expressed at high levels on some peripheral blood and cortical thymic T-cells and at low levels on bone marrow cells, medullary thymocytes and megakaryocytes (Bernard *et al.*, 1984).

In more recent studies, Sandberg and colleagues (1991b) have considered the possibility that NIPC may represent either stem cells or progenitor cells. This possibility was supported by the light scatter profile seen on flow cytometric analysis, which was similar to that seen for stem and progenitor cells, namely they had a high forward and low to intermediate orthogonal light scattering. However, after sorting for cells positive for a variety of hematopoietic or stem cell markers, followed by induction of IFN with HSV and *in situ* hybridization for IFN- α mRNA, the NIPC cells were found to be negative for CD34 (which is a stem cell marker), CD33 (on myeloid progenitor cells), CD10 (on lymphoid progenitor cells) or CD7 or CD19, which are found on T- and B-cell precursors, respectively. A summary of the cell surface markers on NIPC is shown in Table 1.

Although HSV remains the best studied of viruses that induce IFN production by NIPC, it is clear that a low frequency, nonadherent, non-B, non-T, non-NK cell population is the primary (or at least a major) producer of IFN in response to many viruses. Although strict definition of the responding populations will, to some measure, depend upon unique characterization of the NIPC, a number of viruses can be tentatively identified as inducers of IFN from NIPC. These viruses include both DNA and RNA viruses and share the properties of expressing viral glycoproteins in the viral envelope (Table 2).

2.2. RELATIONSHIP OF NIPC TO DENDRITIC CELLS

The low frequency of NIPC among PBMC and their expression of MHC Class II antigens led to the suggestion that NIPC may represent circulating peripheral blood dendritic cells (Perussia *et al.*, 1985). In support of this hypothesis was our ability to enrich for NIPC (Feldman and Fitzgerald-Bocarsly, 1990) using a protocol originally developed to enrich for dendritic cells (Rasanan *et al.*, 1988). However, the enriched populations of NIPC remained rather heterogeneous and although electron micrographs revealed numerous cells with veiled cytoplasmic projections typical of dendritic cells, there were many other cell types visible in the electron micrographs as well. Chehimi *et al.* (1989) examined the relationship of NIPC induced by cytomegalovirus (CMV) to dendritic cells and found that although the NIPC expressed the D44 antigen, enriched dendritic cells that stimulated mixed lymphocyte reactions were D44 negative. However, like many protocols for obtaining highly enriched dendritic cells, the Chehimi protocol required extensive culturing of the cell populations. NIPC function is well documented to be sensitive to *in vitro* culture (Trinchieri *et al.*, 1978b) and even nondendritic cell-enriched populations would demonstrate decreased or absent IFN production under these culture conditions. Therefore, it is possible that the dendritic cells enriched by Chehimi *et al.* have retained the ability to present antigen but can no longer produce IFN. Along these lines, it has been demonstrated that dendritic cells lose their ability to

TABLE I. *Phenotypic Characterization of Human Peripheral Blood NIPC*

Marker	Cells expressing	Present on NIPC?	References
General			
MHC-Class I	All nucleated	Yes	Fitzgerald-Bocarsly <i>et al.</i> , 1988
MHC-Class II			
HLA-DR	B cells, monocytes, others	Yes	Abb <i>et al.</i> , 1983; Perussia <i>et al.</i> , 1985; Bandyopadhyay <i>et al.</i> , 1986; Fitzgerald-Bocarsly <i>et al.</i> , 1988
HLA-DQ	B cells, monocytes, others	Yes	Sandberg <i>et al.</i> , 1989
HLA-DP	B-cells, monocytes, others	Yes	Sandberg <i>et al.</i> , 1989
Nylon wool adherence	B cells, monocytes	No	Fitzgerald-Bocarsly <i>et al.</i> , 1988
Plastic adherence	Monocytes	No	Fitzgerald-Bocarsly <i>et al.</i> , 1988
Esterase staining	Monocytes	No	Fitzgerald-Bocarsly <i>et al.</i> , 1988
Phagocytosis	Monocytes, neutrophils	No	Feldman and Fitzgerald-Bocarsly, 1990; Sandberg <i>et al.</i> , 1990
Lymphoid associated			
CD2	T cells (sheep red blood receptor)	No	Trinchieri <i>et al.</i> , 1978a; Peter <i>et al.</i> , 1980; Perussia <i>et al.</i> , 1985
CD3	T cells (component of T cell receptor)	No	Fitzgerald-Bocarsly <i>et al.</i> , 1988; Sandberg <i>et al.</i> , 1989; Feldman and Fitzgerald-Bocarsly, 1990
CD4	Helper T cells, monocytes	Yes—weak No	Sandberg <i>et al.</i> , 1990 Perussia <i>et al.</i> , 1985; Fitzgerald-Bocarsly <i>et al.</i> , 1988
CD7	T cell subset	No	Sandberg <i>et al.</i> , 1991b
CD8	Cytotoxic/suppressor T cells	No	Perussia <i>et al.</i> , 1985
CD16	NK cells	No	Perussia <i>et al.</i> , 1985; Bandyopadhyay <i>et al.</i> , 1986; Fitzgerald-Bocarsly <i>et al.</i> , 1988
CD19	B cells	No	Kurane and Ennis, 1987; Sandberg <i>et al.</i> , 1991b
CD38	Plasma cells, activated T	No	Rönblom <i>et al.</i> , 1983b; Fitzgerald-Bocarsly <i>et al.</i> , 1988
CD56	NK cells	No	Fitzgerald-Bocarsly <i>et al.</i> , 1988; Feldman and Fitzgerald-Bocarsly, 1990
CD57	NK cells, subset of T cells	No	Abb <i>et al.</i> , 1983; Rönblom, <i>et al.</i> , 1983b; Fitzgerald-Bocarsly <i>et al.</i> , 1988
μ chain	B cells	No	Peter <i>et al.</i> , 1980
κ chain	B cells	No	Peter <i>et al.</i> , 1980; Sandberg <i>et al.</i> , 1989
D44	T cell subset, others	Yes	Lebon <i>et al.</i> , 1982; Chehimi <i>et al.</i> , 1989
Myeloid associated			
CD11b	Granulocytes, monocytes, NK	No	Fitzgerald-Bocarsly <i>et al.</i> , 1988; Sandberg <i>et al.</i> , 1991b
CD11c	Monocytes, granulocytes, NK	No	Sandberg <i>et al.</i> , 1989
CD13	Monocytes, granulocytes	No	Sandberg <i>et al.</i> , 1991b
CD14	Monocytes	No Yes (some)	Sandberg <i>et al.</i> , 1991b unpublished results
CD15	Granulocytes	No	Sandberg <i>et al.</i> , 1991b
CD36	Monocytes, platelets (thrombospondin receptor)	Yes (some)	Sandberg <i>et al.</i> , 1989
Progenitor cells			
CD10	Lymphoid progenitors, some B cells	No	Sandberg <i>et al.</i> , 1991b
CD33	Monocytes, myeloid progenitors	No	Sandberg <i>et al.</i> , 1991b
CD34	Hematopoietic stem cells	No	Sandberg <i>et al.</i> , 1991b

TABLE 2. *Viruses Capable of Inducing IFN Production by NIPC*

Virus	References
DNA viruses	
HSV	Perussia <i>et al.</i> , 1985; Fitzgerald-Bocarsly <i>et al.</i> , 1988; Feldman and Fitzgerald-Bocarsly, 1990; Sandberg <i>et al.</i> , 1990
CMV	Bandyopadhyay <i>et al.</i> , 1986
Human herpes virus 6	Kikuta <i>et al.</i> , 1990
RNA viruses	
HIV	Capobianchi <i>et al.</i> , 1988a; Gendelman <i>et al.</i> , 1992
TGEV (for pigs)	Charley and Laude, 1988; Laude <i>et al.</i> , 1992
Sendai	Perussia <i>et al.</i> , 1985; Rosztóczy and Papós, 1989; unpublished results
VSV	Fitzgerald-Bocarsly <i>et al.</i> , 1988; Howell and Fitzgerald-Bocarsly, 1991
Newcastle disease virus	Perussia <i>et al.</i> , 1985; Fernandez <i>et al.</i> , 1986; unpublished results
Influenza	Perussia <i>et al.</i> , 1985; unpublished results
Mumps	Kato and Minagawa, 1981; Fernandez <i>et al.</i> , 1986
Dengue	Kurane and Ennis, 1987

process, but not to present, antigen following overnight culture, suggesting some, but not other, properties of these cells are culture sensitive. Similarly, treatment of dendritic cells with the lysosomotropic drug chloroquine was shown to inhibit antigen processing but not presentation by dendritic cells; interestingly, IFN- α production by NIPC is also sensitive to chloroquine treatment (Lebon, 1985, unpublished results). In addition, peripheral blood dendritic cells are known to be a heterogeneous population, with cells able to serve as antigen-presenting cells being derived from both light density and heavy density cells on Percoll gradients. Therefore, it remains a possibility that the light-density NIPC represent a subpopulation of, or are related to, dendritic cells. The lack of known unique cell surface markers for either dendritic cells or NIPC leaves the question of the relationship of these two populations unclear. In support of the possibility that NIPC are among the dendritic cell population, Ferbas and Rinaldo (1992) have recently reported that cells purified by flow cytometry for dendritocytes (i.e. cells with dendritic morphology) also express dramatically increased IFN- α levels.

An alternate hypothesis is that the NIPC are independent of any of the lymphoid, myeloid or dendritic cell lineages and are not hematopoietic stem cells or progenitors, but rather represent a unique lineage. Again, further resolution of this issue awaits development of better isolation techniques and/or development of monoclonal antibodies specific for NIPC that would allow positive selection of these cells.

2.3. FREQUENCY ESTIMATES FOR IFN-PRODUCING CELLS

The majority of IFN production in response to HSV can be observed to be mediated by cells from light density Percoll gradient fractions, which represent approximately 1% of the nonadherent PBMC placed on the gradients (Fitzgerald-Bocarsly *et al.*, 1988). More accurate estimates of the frequency of these IFN-producing cells have come from studies using limiting dilution assays, *in situ* hybridization, immunocytochemistry or ELISpot assays. In the limiting dilution assay, Rönnblum and Alm (1982) tested the ability of HSV-stimulated PBMC to produce IFN, which would protect microtiter wells of MDBK against vesicular stomatitis virus attack. In these studies, it was observed that the IFN-producing cells were present in low frequency in the peripheral blood (less than 1:100); moreover, the single hit kinetics of this limiting dilution assay indicated that a single IFN-producing cell was capable of producing enough IFN to protect an entire microtiter well, i.e. >0.25 IU/cell. In *in situ* hybridization studies for IFN mRNA, Gobl *et al.* (1988) observed that approximately 1:1000 PBMC stimulated with HSV expressed IFN- α 2 mRNA, in contrast to approximately 1:100 cells, which responded to Sendai virus. Although the HSV-induced PBMC produced IFN at a lower frequency than did the Sendai-induced cells, the bulk IFN

production in culture was typically similar with these two viruses, leading to the conclusion that each HSV-responding cell produces much more IFN than the Sendai-responding cells. This conclusion is supported by the observation that in the *in situ* hybridization studies for IFN- α 2 mRNA (Gobl *et al.*, 1988), the greatest fraction of HSV-responding cells contained >100 silver grains in autoradiography, whereas the Sendai-responding cells contained >20 grains.

In order to study better the cells producing IFN in response to HSV, we sought to purify IFN-producing cells using a protocol designed to enrich for peripheral blood dendritic cells (Rasanan *et al.*, 1988; Feldman and Fitzgerald-Bocarsly, 1990). This protocol involved depleting PBMC of monocytes followed by centrifugation of the cells on 48% Percoll density gradients. The light-density cells were then further depleted of monocytes (since residual monocytes re-enriched on the Percoll density gradients) by carbonyl iron ingestion followed by magnetic depletion. NK cells (which also enrich in the light-density Percoll gradient fractions) and T cells were then removed by treatment with monoclonal antibodies followed by magnetic immunobeads coated with antimouse IgG antibodies and magnetic depletion. Populations collected at each stage were stimulated with UV-inactivated HSV-1 for 6 hr and immunocytochemistry was performed to visualize intracellular IFN- α and, in parallel, were stimulated for 14 hr for determination of IFN- α production. Our results indicated that among the unseparated PBMC, only occasional cells could be positively stained for intracellular IFN following stimulation with HSV, to yield a frequency of <1:1000. The frequency of IFN-positive cells was found to increase with our sequential enrichment protocol, with approximately 12–13% of the cells in the final population being positive for intracellular IFN following staining with anti-IFN, representing a >125-fold enrichment of the IFN-producing cells (Fig. 1). From these studies, each NIPC was calculated to produce between 1 and 2 IU of IFN, numbers in close agreement with estimates of Rönblom and Alm (1982). In the anti-IFN stained, HSV-stimulated IFN-producing cell-enriched populations, the IFN-producing cells were found to have the interesting property of clustering together (Feldman and Fitzgerald-Bocarsly, 1990; see Fig. 2); such clustering was also observed in microtiter wells of viable PBMC (Feldman, M. *et al.*, 1992) and increased with enrichment of the IFN-producing cells. The

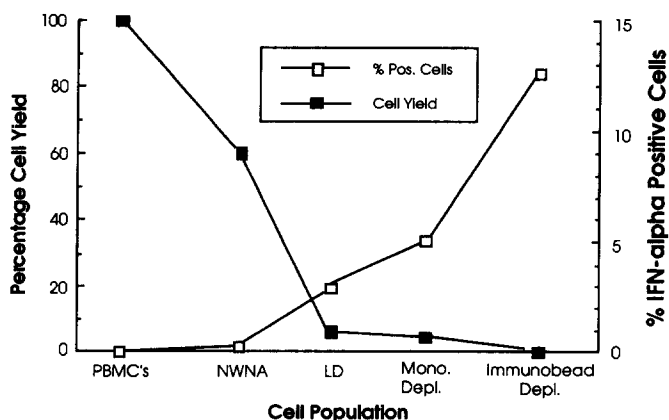


FIG. 1. Prevalence of IFN- α -producing cells and cell yield of sequentially enriched PBMC populations. PBMC from healthy individuals were sequentially fractionated by passage through nylon wool columns (nylon wool nonadherent, NWNA), followed by centrifugation on a Percoll density gradient (light density, LD). The LD population was depleted of monocytes using carbonyl iron plus magnet (monocyte depleted, Mono. Depl.) and CD3⁺ T cells and CD56⁺ NK cells were removed by indirect immunomagnetic bead depletion (CD3⁺/CD56⁺ depleted, Immunobead Depl.). Each population was stimulated with UV-inactivated HSV-1 for 6 hr, cytocentrifuge preparations were made, then immunocytochemistry was performed using a sheep anti-human IFN- α antiserum (kindly provided by Dr S. Pestka) followed by an avidin-biotin peroxidase technique. A minimum of 1000 cells/slide were counted. Data are the mean of two experiments. The range of the data points was <10% for each value. Cell yields were calculated from the original PBMC population. Reproduced from Feldman and Fitzgerald-Bocarsly, 1990, with permission of the copyright holder, Mary Ann Liebert Inc. Publishers, New York.

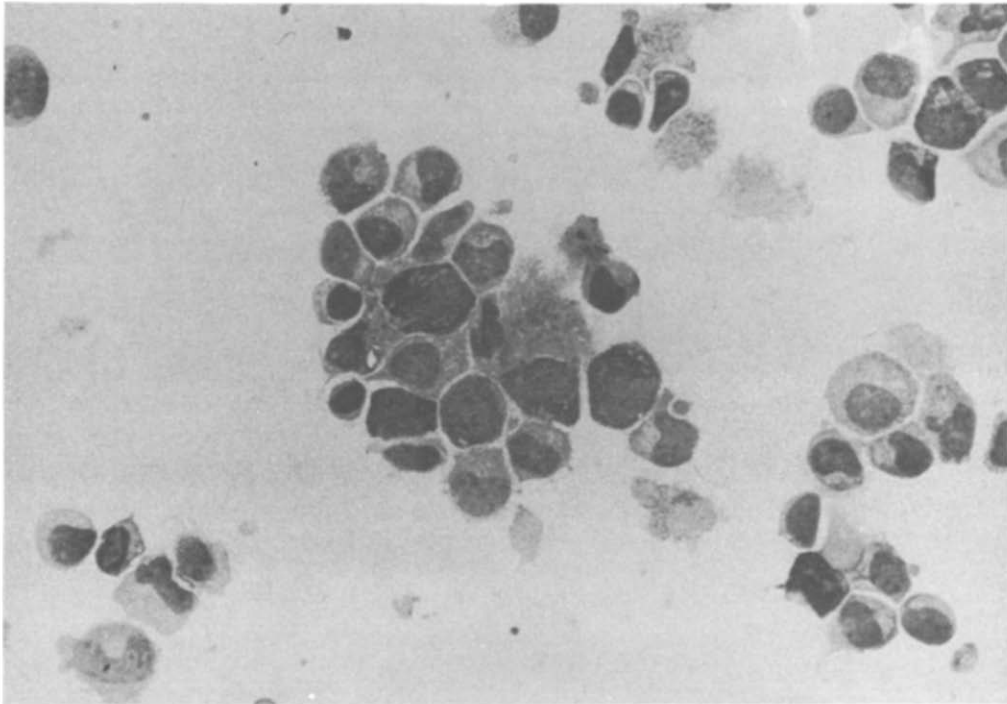


FIG. 2. Immunocytochemistry of sequentially enriched NIPC. Populations of cells enriched for NIPC were prepared as described in Fig. 1, then stimulated for 6 hr with UV-inactivated HSV-1 and immunocytochemistry was performed with a sheep anti-human IFN- α antiserum as described in legend, Fig. 1.

clustering phenomenon, which is also typical of dendritic cells (Inaba and Steinman, 1987), was removed by depleting the populations of HLA-DR⁺ cells. Although the enrichment technique was effective in enriching for IFN-producing cells, it is limited by the still heterogeneous nature of the final populations that were found by flow cytometry to still contain significant numbers of contaminating T cells, B cells, NK cells and macrophages and by the low cell yield obtained in the enrichment protocol, with the final yields being only about 0.5% of the starting PBMC population. Electron micrographs of the enriched populations show interesting patterns, even though the populations are heterogeneous. The micrographs indicate a preponderance of large cells with abundant cytoplasm and a lack of evidence of phagocytosis and the presence of cytoplasmic veils on many of the cells. However, since these electron micrographs did not include staining for cytoplasmic IFN, it is not currently possible to describe the electron-microscopic features of the IFN-producing cells. Because of the low yield of cells following sequential enrichment, further enrichment of significant numbers of the IFN-producing cells by this sort of approach seems unlikely unless an alternate source of mononuclear cells is identified. One such possible source could be normal spleens removed from trauma patients; however, it is not currently known whether the NIPC are found in the human spleen.

An additional method of evaluating the frequency of IFN-producing cells was described by Alm and colleagues (Rönblom *et al.*, 1988; Cederblad and Alm, 1990). This assay utilized the ELISpot technique (or solid-phase immunoplaque assay) for enumerating IFN-producing cells. This technique involves stimulating PBMC with either virus or virus-infected cells, followed by plating them onto nitrocellulose filter-bottomed microtiter wells that have been coated with anti-IFN antibody. The cells are then cultured in the plates to allow IFN release and the IFN is captured by the anti-IFN antibodies. The cells are then washed out of the plate and the plates are treated with a monoclonal antibody to IFN- α followed by treatment with peroxidase conjugated secondary antibody and development with a diaminobenzidine tetrahydrochloride substrate. Captured IFN yields spots, each of which represents the IFN produced by an individual IFN-producing cell. These spots can then be enumerated and frequency of IFN-producing cells determined. This technique

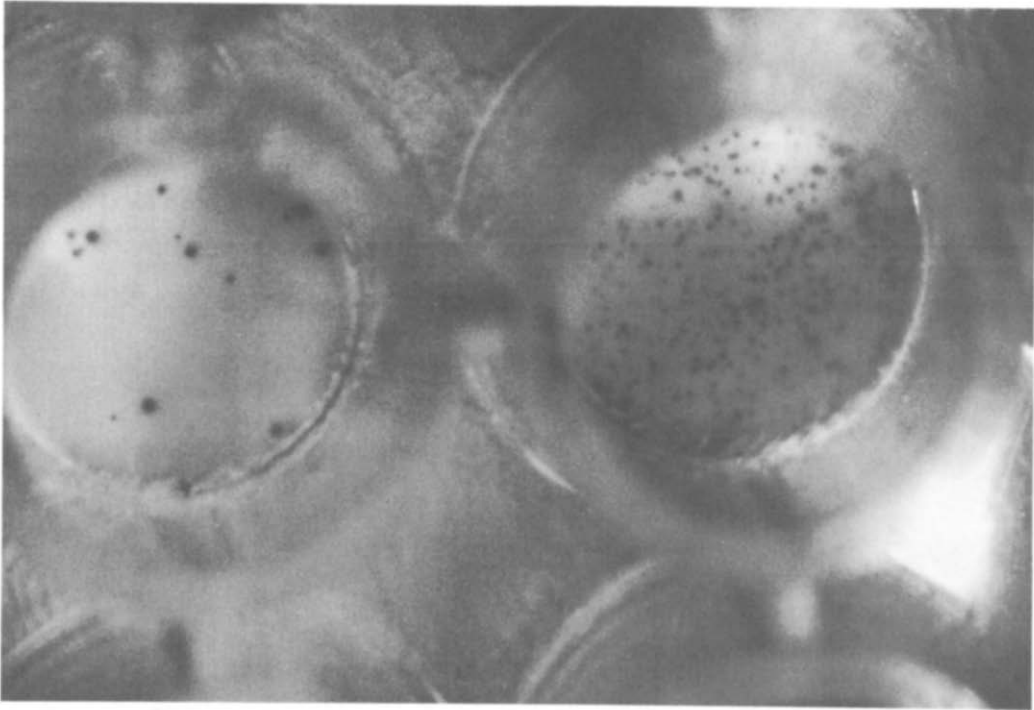


FIG. 3. ELISpot frequency analysis of IFN-producing cells responding to HSV and Sendai virus. PBMC from a healthy donor were stimulated with UV-inactivated HSV (left) or Sendai virus (right) for 6 hr, then incubated for 12 hr in millititer plates coated with a polyclonal bovine anti-human leucocyte IFN (AS94). Plaques were developed with a monoclonal antibody to human IFN- α (LO22) followed by a horseradish peroxidase conjugated rabbit anti-mouse IgG antibody and development with DAB substrate. For this donor, the frequencies of IFN-producing cells for HSV and Sendai were 1/2415 and 1/60, respectively, with IFN production/IFN-producing cell values of 0.72 IU and 0.18 IU, respectively.

has provided similar estimates of IFN-producing cell frequency as the techniques mentioned above, with our recent study demonstrating that the healthy control population had a mean frequency of 1:703 IFN-producing cells among the PBMC, with each IFN-producing cell making on average 1.9 IU of IFN.* This technique has the advantage of yielding more easily obtained data than either the limiting dilution assays, the *in situ* hybridization studies or by immunocytochemistry. The latter two assays require microscopic counting of individual cells and the former requires plating of multiple microtiter plates for each sample tested. In contrast, the ELISpot assay yields data rapidly and is well suited for evaluation of IFN-producing cell frequency of patient populations, since only about $1-2 \times 10^6$ cells are needed for each patient sample. The data we have obtained using this assay with PBMC from HIV-infected patients is described in Section 6.1.

We have also recently utilized the ELISpot assay to obtain frequencies of IFN-producing cells in PBMC populations stimulated with Sendai virus (Feldman, S. *et al.*, 1992). Again, in agreement with the *in situ* hybridization studies of Gobl *et al.* (1988), the IFN-producing cells induced by Sendai virus are much more frequent than those observed for HSV (our mean frequencies are approximately 2.0 vs 0.1%, respectively) and each Sendai virus-responsive cell is calculated to produce approximately 0.15 IU of IFN vs >10-fold higher values for HSV-responsive cells. This lower production of IFN by each Sendai-responsive cell is also reflected visually in the sizes of the spots produced by HSV vs Sendai in the ELISpot assay, with Sendai spots being characteristically more numerous but much smaller than the HSV-induced spots (Fig. 3).

*Howell, D., Feldman, S., Kloser, P. and Fitzgerald-Bocarsly, P. (1993) Decreased frequency of natural interferon producing cells in peripheral blood of patients with the acquired immune deficiency syndrome. Submitted for publication.

3. REQUIREMENTS FOR IFN GENE EXPRESSION IN NIPC

Analysis of IFN produced by NIPC in response to HSV or CMV has indicated that virtually all of the IFN could be neutralized by polyclonal antisera to IFN- α (Fitzgerald *et al.*, 1982; Bishop *et al.*, 1983; Bandyopadhyay *et al.*, 1986). In order to evaluate which IFN genes are expressed in these systems, a number of approaches have been taken. Gobl and colleagues (1988) have demonstrated by *in situ* hybridization that NIPC produce IFN- α 2 and IFN- β mRNA in response to glutaraldehyde-fixed HSV-WISH cells. Peak message was observed approximately 6 hr from the start of induction, similar to data previously reported for induction of fibroblasts (Cavaliere *et al.*, 1977b) and peripheral blood leukocytes (Familletti *et al.*, 1981). Sendai virus-induced leukocytes also made both IFN- α 2 and - β mRNA, but the peak IFN- β message appeared earlier than the IFN- α mRNA (4 vs 6 hr, respectively). Moreover, the Sendai virus-induced leukocytes, which, as described in Section 1.2, are primarily monocytic, showed fewer grains/cells in autoradiograms than did the HSV-induced cells, suggesting that the greater production of IFN/cell observed for NIPC vs monocytes can at least partially be accounted for by increased transcription of the IFN genes. We have studied IFN mRNA production by S1-mapping in populations sequentially enriched for NIPC in response to both Sendai virus and UV-inactivated HSV. Evidence for transcription of IFN- α 1, - α 2, - α 7 and - β mRNA, but not IFN- ω or IFN- γ , was found (D. Howell, J. Hiscott and P. Fitzgerald-Bocarsly, manuscript in preparation).

The observation of IFN- β mRNA production in NIPC in response to HSV is interesting since most or all of the IFN produced by these cells could be neutralized by antiserum to IFN- α . Cederblad and colleagues were able to demonstrate very low levels of IFN- β production by NIPC using an IFN- β -specific ELISA assay: approximately 2–5% of the IFN produced in response to HSV was found to be IFN- β (Cederblad and Alm, 1991). The ability of the NIH polyclonal antiserum to IFN- α to neutralize all of the IFN produced in response to HSV can probably be explained by the small amount of cross-reactivity with IFN- β reported for this antiserum. This cross-reactivity may reflect reactivity with a common epitope shared between IFN- α and - β , which accounts for their ability to bind to the same receptor (Kontsek *et al.*, 1990). Even though IFN- β message is transcribed, it appears that the majority of IFN- β mRNA is not translated. This observation of post-transcriptional control of IFN- β is not without precedence in that Hiscott *et al.* (1984) have reported a similar situation in Namalwa cells stimulated with Sendai virus. However, Namalwa cells were previously shown to produce some IFN- β (Cavaliere *et al.*, 1977a).

As described above, IFN- β mRNA expression by monocytes in response to Sendai virus occurs without a lag phase, whereas IFN- α mRNA appears somewhat later (Gobl *et al.*, 1988). In NIPC, both IFN- α and IFN- β mRNA production occurs following a substantial lag period, with peak mRNA levels occurring at about 6 hr. For Sendai virus-induced cells, the majority of the IFN-producing cells were found to express both the IFN- α and - β genes, while approximately 30% produced only the IFN- β genes. These kinetics of expression of IFN- α and - β mRNA in Sendai virus-induced monocytes suggested to Gobl and colleagues (1988) that differential rather than coordinate regulation of IFN- α and IFN- β genes was occurring in the cells. To test this hypothesis, they examined the effects of protein synthesis inhibitors on IFN- α and IFN- β mRNA synthesis. IFN- α , but not IFN- β , mRNA synthesis by monocytes was found to be sensitive to cycloheximide treatment (Gobl *et al.*, 1992). When similar studies were carried out with HSV-induced NIPC cells, both IFN- α and IFN- β mRNA were found to be sensitive to cycloheximide treatment (Cederblad *et al.*, 1991). Nuclear run-on experiments indicated that the sensitivity to cycloheximide was due to decreased transcription of the genes rather than to increased turnover of the mRNA.

We have recently observed that the arginine analog canavanine is able to inhibit IFN production by NIPC in response to HSV (Feldman, M. *et al.*, 1992). Carry-over of canavanine into the IFN bioassays and prevention of the induction of the antiviral state did not account for the decreased IFN measured, since addition of canavanine directly to IFN bioassays had no effect. One possible explanation for the failure to detect IFN in the bioassay was that an arginine-substituted IFN molecule was unable to bind to the IFN receptor and/or transduce appropriate signals. The presence of a crucial arginine residue at position 23 on IFN- α has been reported (Meager and Berg, 1986). Supernatants generated in the presence of canavanine not only lacked antiviral activity, but were also unable to stimulate NK cell activity (Feldman, M. *et al.*, 1992). In addition, the

canavanine-incubated cells did not contain any immunologically reactive species in either IFN- α ELISA or ELISpot assays (Feldman, S. and Fitzgerald-Bocarsly, 1992; S. Feldman and P. Fitzgerald-Bocarsly, manuscript in preparation). We, therefore, looked for the effect of canavanine on IFN mRNA expression in HSV-induced cells by reverse transcriptase polymerase chain reaction for IFN- α 1, - α 2 and - β mRNA. Lowered levels of transcripts for each of the virally induced mRNAs generated in the presence of canavanine were observed (Feldman, S. and Fitzgerald-Bocarsly, 1992). These data support the studies of Gobl and Cederblad demonstrating a requirement for protein synthesis for NIPC mRNA transcription.

The mechanism by which IFN message transcription by NIPC is sensitive to cycloheximide or canavanine is unknown. However, studies of Alm and colleagues have suggested that treatment of the leukocytes with conditioned media or interleukin 3 and/or granulocyte-macrophage colony stimulating factor can overcome the requirement for *de novo* protein synthesis in the NIPC (Cederblad and Alm, 1991; Cederblad *et al.*, 1991). These observations suggest that optimal production of IFN by the NIPC requires another signal to be presented to the cells. Whether the NIPC cells themselves produce the other signal, which then induces the IFN gene expression in an autocrine fashion, or whether the necessary protein synthesis occurs in a second cell, with the cytokines acting in a paracrine manner, remains to be determined. The current inability to prepare highly purified populations of NIPC limits the ability to carry out the appropriate experiments. However, observations by Alm's group that the optimal frequency of NIPC is not seen unless the responding cells are at a certain concentration or are provided with cytokines and/or feeder cells suggests that another cell type may be providing an accessory factor to the NIPC (Cederblad and Alm, 1991; Cederblad *et al.*, 1991). However, if another cell type was necessary, then it would be expected that depletion of other cells should reduce IFN production by NIPC, a phenomenon that is not observed. The possibility of a requirement for production of another factor for optimal NIPC function may account for the necessity of providing feeder cells and/or cytokines to small numbers of sorted cells in order to see IFN generation (Sandberg *et al.*, 1991b).

In recent experiments, Sandberg and colleagues have studied the role of protein kinase C in production of IFN- α and - β by monocytes and NIPC. The phorbol ester, phorbol myristate acetate (PMA), which activates protein kinase C, was found to inhibit efficiently the *in vitro* IFN- α and - β responses in human blood monocytes stimulated with Sendai virus, as well as in NIPC induced by glutaraldehyde-fixed HSV-WISH cells (Sandberg *et al.*, 1991a). The PMA inhibition of IFN production was correlated with decreased levels of IFN- α and - β mRNA expression. The protein kinase inhibitor staurosporine prevented the PMA-mediated inhibition of IFN expression, suggesting a role for protein kinase C in the inhibition of IFN- α/β responses. In contrast, we have observed that staurosporin (which inhibits both protein kinase C and tyrosine kinase) and herbimycin (which is specific for tyrosine kinase) are both able to inhibit IFN production by NIPC in response to UV-inactivated HSV, implicating a role for tyrosine kinase and/or protein kinase C in activation of NIPC (M. Feldman, Q. Li and P. Fitzgerald-Bocarsly, unpublished observations). In addition, IFN- α production was sensitive to calcium chelation with EGTA (Feldman, M. and Fitzgerald-Bocarsly, 1992). It will be important to determine whether either of these kinases are directly involved in transducing a signal from the cell surface to the NIPC to begin IFN gene expression, or whether their role is less direct.

4. MECHANISM OF IFN INDUCTION IN NIPC

The mechanism by which viruses induce IFN production by leukocytes has received attention by several groups in recent years. IFN production is known to be induced in response to double-stranded RNA, which occurs during the replicative cycle of many (particularly RNA) viruses or to synthetic polyribonucleotides, such as poly I:poly C (Torrence and De Clercq, 1981). Capobianchi *et al.* (1988b) have reported that production of IFN in response to HSV requires presentation of viral protein by infected cells, with free HSV being a very poor inducer of IFN. However, we and others have observed that NIPC are efficient producers of IFN in response to live HSV, UV-inactivated HSV, as well as to live, HSV-infected fibroblasts or glutaraldehyde fixed,

HSV-infected cells, indicating that HSV replication and/or gene expression is not required for IFN induction in NIPC (Feldman *et al.*, 1990; Fitzgerald *et al.*, 1982; Rönnblum *et al.*, 1988).

The possibility that HSV viral glycoproteins are responsible for induction of IFN- α in NIPC was proposed by Lebon (1985), who observed that antibody to HSV glycoprotein D (gD) was able to block IFN production in response to HSV-infected, glutaraldehyde fixed cells. Similarly, we have observed that polyclonal antisera to HSV is also able to block production of IFN by PBMC to HSV-infected fibroblasts (Fitzgerald-Bocarsly *et al.*, 1991). The viral glycoprotein gD is required for internalization of HSV virions into cells; in the absence of gD, the virus is able to bind but not to internalize or replicate (Ligal and Johnson, 1988). However, since the IFN-inducing cells used by Lebon were glutaraldehyde fixed, it is unlikely that the critical block is at the level of internalization of the HSV by the responding NIPC. Other studies have suggested a role for viral glycoproteins in induction of IFN. Ito *et al.* (1978) reported that reconstituted Sendai virus membranes, which were free of viral nucleic acids but contained glycoproteins, could induce IFN production in mice, a result which we have recently reproduced using human PBMC (N. Patel, S. Gould-Fogerite and P. Fitzgerald-Bocarsly, unpublished results). Similarly, nucleic acid-free reconstituted influenza virus envelopes were recently shown to induce IFN in PBMC (Yasuda *et al.*, 1992). Soluble gp120 envelope protein of HIV recently has been reported to induce IFN- α in PBMC (Capobianchi *et al.*, 1992); however, very high levels of the protein, corresponding to three or four logs more than found in HIV stock solutions, were required for this induction. It is possible that this requirement for high levels of soluble gp120 may reflect a relative inefficiency of soluble protein (as opposed to membrane-expressed protein) to induce IFN.

The concept of interferogenic proteins and epitopes has been introduced by Charley and colleagues studying the transmissible gastroenteritis virus (TGEV), which infects pigs (Charley and Laude, 1988; Laude *et al.*, 1992). Recently, they have described a site on the N-terminal, extracellular portion of the viral glycoprotein M, which when mutated renders the virus unable to induce IFN in porcine PBMC. Monoclonal antibodies directed against the glycoprotein M of TGEV were also able to block the ability of the virus to induce IFN. A possible role for glycosylation at the critical sites is suggested by the decreased ability of TGEV particles treated with endoglycosidase PNGase F to induce IFN by PBMC (Charley and Laude, 1988). It is possible that sugar residues on viral glycoproteins bind to receptors on the NIPC and transduce the signal for IFN induction to the nucleus of these cells. Although the hypothesis is clearly preliminary and warrants further study, it is intriguing to consider whether different viral glycoproteins could bind to a given receptor on NIPC. Since glycoproteins on the surface of noninfected, autologous cells do not induce IFN and not all viral glycoproteins appear to be interferogenic, it will also be interesting to determine how self vs nonself glycoproteins are distinguished by the IFN-producing cells.

5. ROLE OF NIPC IN NATURAL KILLER CELL ACTIVITY AGAINST VIRALLY INFECTED TARGETS

Co-operation between cells of the immune system is crucial for optimal host defense. One example of cell-cell co-operation, which may involve NIPC, is in the lysis of virally infected targets by human NK cells. NK cells express an Fc receptor for immunoglobulin G on their surfaces and can be positively or negatively selected, based on the expression of this molecule. Treatment of PBMC with monoclonal antibody against CD16 plus complement abrogates kill of both K562 erythroleukemia targets as well as against HSV-infected fibroblasts. When NK cells are positively selected by cell sorting of PBMC labeled with anti-CD16-FITC, they demonstrate increased lysis of K562 targets, whereas the CD16-negative fractions fail to lyse the tumor targets (Fitzgerald-Bocarsly *et al.*, 1989a). In contrast to the above results, neither the positively selected NK cells nor the NK-depleted fractions are able to kill fibroblasts infected with HSV or CMV. However, when the NK-depleted populations are added back to the NK-enriched populations, the ability of the NK cells to lyse the virally infected targets is restored, leading to the conclusion that the presence of a CD16⁻ accessory cell (AC) population is required for the lysis of the virally infected fibroblasts but not K562 cells. AC dependence of NK lysis has been shown for fibroblast targets infected with

DNA viruses in the herpesvirus family, including HSV, CMV and *Varicella zoster* virus (Bandyopadhyay *et al.*, 1986; Oh *et al.*, 1987; Fitzgerald-Bocarsly *et al.*, 1989a), as well as for lysis of targets infected with the RNA viruses vesicular stomatitis virus (Howell and Fitzgerald-Bocarsly, 1991) or HIV-1 (Bandyopadhyay *et al.*, 1990). We have screened approximately 17 infected and noninfected targets for AC-dependent NK cell lysis and found that all of the nonvirally infected tumor cells are killed in the absence of ACs, whereas the virally infected fibroblast or solid tumor cell targets require AC participation (Howell and Fitzgerald-Bocarsly, 1991). An exception to the rule of AC requirement for NK lysis of cells infected with viruses was found for lysis of HSV-infected lymphoblastoid cells; lysis of these target cells was found to be only partially dependent upon the presence of CD16⁻ ACs.

The requirement for ACs for lysis of virally infected targets can be overcome by addition of known NK activators, such as IFN- α or IL-2 to AC-depleted NK populations (Fitzgerald-Bocarsly *et al.*, 1989a) or by the addition of supernatant generated from co-cultures of PBMC and virally infected targets (Bandyopadhyay *et al.*, 1986). The NK augmenting factor in these supernatants has been shown to be IFN- α by the ability of NK augmenting activity to be neutralized by anti-IFN- α antisera.

Characterization of the AC indicates that they share many, if not all, of the properties of the NIPC: the AC and NIPC are both light density, HLA-DR⁺ (MHC Class II) cells negative for T-cell, B-cell and NK-cell markers and are found to lack most properties characteristic of monocytes, i.e. they are plastic nonadherent, nonphagocytic and negative for the monocyte determinant OKM2 (Bandyopadhyay *et al.*, 1986; Fitzgerald-Bocarsly *et al.*, 1989a; Feldman and Fitzgerald-Bocarsly, 1990; Feldman, M. *et al.*, 1992). Removal of HLA-DR⁺ cells eliminates both lysis of HSV-infected fibroblasts as well as IFN generation during the NK assays, but has no effect on lysis of K562 targets. Sequential enrichment of NIPC also leads to enrichment of AC activity, with AC being required in only small numbers to permit lysis of the infected targets (Fitzgerald-Bocarsly *et al.*, 1989a; Feldman and Fitzgerald-Bocarsly, 1990; Feldman, M. *et al.*, 1992). Further similarities between the NIPC and AC populations include their sensitivity to emetine, an irreversible inhibitor of protein synthesis (Feldman, M. *et al.*, 1992) and to chloroquine, which disrupts acidic compartments by raising the pH (Lebon, 1985, unpublished results). Although the NIPC are prime candidates for the AC population, we have recently found that positively selected CD14-bearing cells are also able to function as AC for NK lysis (M. Milone and P. Fitzgerald-Bocarsly, unpublished results). As described in Section 2.1, these CD14⁺ cells are also capable of producing IFN- α in response to HSV-infected cells or UV-inactivated HSV. These results may reflect either redundant populations of cells that are able to carry out these functions, or alternately, that NIPC may express CD14 at some or all stages of their differentiation.

Although it is reasonable to hypothesize that NIPC function as ACs via the production of IFN- α , which then serves to activate the NK cells, our other evidence indicates that AC function can, in some cases, occur in the absence of detectable IFN. For example, when polyclonal sheep anti-IFN- α antiserum was added to NK assays to neutralize all the IFN generated in response to HSV-infected fibroblasts (Fitzgerald *et al.*, 1982; Bishop *et al.*, 1983), there was no effect on NK activity. These results might be interpreted to mean that at optimal ratios of AC to NK cells the anti-IFN antiserum is unable to get in between NK cell:AC pairs and neutralizes the IFN that is being communicated. In recent studies, we extended these observations by testing the ability of either polyclonal sheep antihuman IFN- α or bovine antihuman IFN- α to inhibit NK activity against HSV-infected fibroblasts when serially decreasing numbers of ACs were added to a fixed number of NK cells. We reasoned that at low AC to NK cell ratios, the IFN would have to diffuse across greater intercellular distances to reach the NK cells and would, therefore, be more susceptible to neutralization. However, regardless of the AC:NK ratio, the sheep antiserum failed to inhibit NK activity against the HSV-infected fibroblasts, even though it was able to neutralize all the IFN produced during the NK assay, suggesting an IFN-independent mechanism of AC function (Feldman, M. *et al.*, 1992). Studies with the arginine analog canavanine provide a second line of evidence that AC-dependent NK lysis can occur in the absence of IFN (Feldman, M. *et al.*, 1992). When NK assays were performed in the presence of canavanine, IFN- α production was completely inhibited, whereas NK activity was only partially reduced. The remaining NK activity was found to be AC dependent, since depletion of HLA-DR⁺ cells removed the remaining lytic activity.

Supernatants generated in the presence of canavanine did not contain any cytokines that could augment NK activity when added to NK cells, arguing against a soluble factor being responsible for the AC activity.

A third line of evidence supporting an IFN-independent mechanism of AC function has come from our studies with NK activity in AIDS patients (Howell *et al.*, 1993). Previously, we have demonstrated that patients with advanced AIDS have a pronounced deficiency in their ability to produce IFN- α in response to HSV (Lopez *et al.*, 1983; Siegal *et al.*, 1986; also see Section 6.1). In our recent studies, ACs from IFN-deficient AIDS patients were found to provide AC function in the absence of IFN- α production. Together, these results suggest that a cell similar or identical to the NIPC is required for providing accessory help to NK cells for the lysis of most virally infected targets; however, IFN released by these cells does not appear to be required for the functioning of these ACs. Therefore, AC activity may either be an independent function of the NIPC or, alternately, may be the function of a similar, but distinct cellular population. Again, as for many of the unanswered questions described above, resolution of this question may require identification of unique cell surface markers present on the AC and/or the NIPC.

6. ROLE OF NIPC IN HUMAN DISEASE

6.1. IFN PRODUCTION IN AIDS

AIDS is characterized by aberrations in many parameters of both the acquired and adaptive immune systems. In addition to infecting CD4⁺ T cells, macrophages and dendritic cells are reported to be major reservoirs of HIV. The IFN system in many patients with AIDS is grossly abnormal, with both *in vivo* and *in vitro* responses showing major deviations from normal. Serum taken from AIDS patients is frequently found to have measurable to high levels of IFN- α (DeStefano *et al.*, 1982). Additionally, there have been reports of circulating IFN- β (Minagawa *et al.*, 1989) in some patients. The presence of serum IFN has been reported to be of prognostic value for monitoring disease progression in patients with AIDS (Buimovici-Klein *et al.*, 1986). The circulating IFN- α is unusual in that it shows an acid lability not typically seen for IFN- α . Acid labile serum IFN- α is also seen in a number of different clinical scenarios, including patients with lupus (Preble *et al.*, 1982; Yee *et al.*, 1989), multiple sclerosis (unpublished results), juvenile arthritis (Arvin and Miller, 1984), Bell's palsy (Jonsson *et al.*, 1989) and some acute viral infections. This serum IFN does not appear to reflect expression of a unique IFN- α gene or genes whose product is intrinsically acid labile, but rather appears to be conferred by other as yet unidentified serum components (Yee *et al.*, 1989). The cellular source of the serum IFN- α in AIDS or other patients is unknown. Tovey and colleagues have performed *in situ* hybridization studies to look for expression of IFN- α genes in tissues taken at autopsy from patients with AIDS, as well as from normal controls. Although they found low constitutive expression of IFN- α genes in many tissues (Tovey *et al.*, 1987), there was no difference between the expression in patients and the controls (Tovey *et al.*, 1992) and the source of the IFN remains unknown. It is possible that the NIPC is the source of the IFN and that their low frequency has made detection impossible.

Several years ago, we observed that patients with AIDS exhibited a deficiency in the ability to produce IFN *in vitro* in response to either UV-HSV or HSV-infected fibroblasts. This deficiency, which was observed in both HIV-1 (Lopez *et al.*, 1983; Siegal *et al.*, 1986) and HIV-2 infection (Fitzgerald-Bocarsly *et al.*, 1989b), was most profound in patients with either concurrent or a history of opportunistic infections and, in fact, was predictive of opportunistic infections during the immediate follow-up period for those HIV⁺ individuals not yet meeting the AIDS case definition (Siegal *et al.*, 1986). The deficiency in IFN- α production was not correlated with levels of CD4⁺ cells in the patients. However, we observed that opportunistic infections did not occur in the patients until both the IFN- α and CD4⁺ cells fell below critical levels (CD4⁺ cells < 300/mm³, IFN- α production < 300 IU/mL). Thus, compromise of these parameters of both the adaptive and the innate immune systems were required for the development of fulminant AIDS, suggesting that there is an overlap in the protective functions of these populations, which is to the individual's advantage. This overlapping function may explain the observations that some HIV⁺ patients with very low helper T cells do surprisingly well for extended periods of time. Deficient

IFN- α production in patients with AIDS has been independently confirmed (Abb and Deinhardt, 1984; Rossol *et al.*, 1989; Voth *et al.*, 1990), with the latter group also reporting, in agreement with our previous report (Siegal *et al.*, 1986), that an advanced deficiency in both CD4⁺ cells and IFN- α production is correlated with disease progression.

The relationship of deficient *in vitro* IFN production and the serum IFN seen in many patients is unclear. In an earlier study, we failed to find a direct correlation between these two parameters; i.e. some patients with serum IFN failed to demonstrate deficient *in vitro* production and some patients with deficient *in vitro* production failed to express IFN in their serum (Lopez *et al.*, 1984).

In order to study whether the decreased IFN production by AIDS patients reflects a decreased frequency of functional NIPC or a decreased production of IFN by each NIPC, we have been utilizing the ELISpot technique to measure the frequency of NIPC in patients with HIV vs healthy controls in response to HSV. We observed that patients with AIDS had a significantly lower NIPC frequency than healthy controls, with some patients having virtually undetectable NIPC.* As would be predicted, those individuals with the most advanced HIV disease had the lowest NIPC frequencies and those with early disease were not statistically different from our control population. In addition to having a lower frequency of NIPC, we also observed that the AIDS patients frequently produced less IFN per NIPC, as calculated from the frequency of NIPC and the IFN produced by the total PBMC population. This decreased IFN per NIPC was also evidenced in the size of the spots produced by the NIPC: many of the AIDS patients produced spots that were quantitatively smaller than those of normal controls, suggesting that less IFN was being released and, hence, captured in the ELISpot assay. Together, these results suggest that there is a turn-off of IFN production in patients with HIV that appears to occur progressively. Further studies, preferably performed longitudinally with a specific group of patients, will be necessary to test this hypothesis. Whether the NIPC actually disappear from the peripheral blood or whether they are physically present but functionally deficient, remains to be determined. For example, it is possible that the NIPC are killed, either directly or indirectly, by HIV. Alternately, the NIPC may be physically present but functionally deficient. If the NIPC turn out to be dendritic cells, it is possible that these, like other dendritic cells, become infected with HIV (Macatonia *et al.*, 1990) and are either killed or rendered dysfunctional. It is also possible that, *in vivo*, the NIPC may be responding to continual stimulus with HIV and/or cytokines being elicited by HIV, leading to a functional exhaustion of the cells. The phenomenon of 'blocking' of subsequent IFN production by pretreatment of cells with high levels of IFN has been reported (reviewed in Stewart, 1981) and may be occurring in the patient NIPC exposed to serum IFN. However, these blocking studies were performed with nonrecombinant IFN preparations and the mechanism by which these IFN preparations were able to block IFN production, or even whether IFN itself or some contaminant is responsible, remains controversial.

An alternate hypothesis regarding the low *in vitro* production of IFN by PBMC from AIDS patients is that the cells are being sequestered in the tissues where they remain functional. These sequestered cells could then be producing the IFN that appears in the serum of the AIDS patients. However, the failure of Tovey *et al.* (1992) to find evidence of upregulated expression of IFN genes in the tissues of patients tends to argue against this possibility. Voth and colleagues (1990) have studied IFN- α mRNA expression from PBMC from AIDS patients and observed decreased levels of gene expression, leading them to conclude that the defect lies at the level of transcription. However, their study has not addressed the possibility that the NIPC are physically absent from the peripheral blood. Studies in our laboratory are currently directed towards looking at the issue of gene expression and at attempting to restore IFN production to the PBMC. Moreover, we are seeking to define a cell-surface marker on the NIPC, which would allow us to directly quantitate these cells.

We have also studied IFN production in HIV-infected patients in response to Sendai virus (Feldman, S. *et al.*, 1992). As described in Section 1.2, Sendai virus primarily induces the monocyte to produce IFN, with a smaller NIPC component. Production of IFN in response to HSV and

*Howell, D., Feldman, S., Kloser, P. and Fitzgerald-Bocarsly, P. (1993) Decreased frequency of natural interferon producing cells in peripheral blood of patients with the acquired immune deficiency syndrome. Submitted for publication.

TABLE 3. Reported Incidence of Aberrant IFN- α Expression in Human Disease

Disease	Serum IFN	Decreased <i>in vitro</i> IFN production?	References
Infectious diseases			
AIDS	Yes	Yes	DeStefano <i>et al.</i> , 1982; Lopez <i>et al.</i> , 1983; Abb and Deinhardt, 1984; Buimovici-Klein <i>et al.</i> , 1986; Siegal <i>et al.</i> , 1986; Fitzgerald-Bocarsly <i>et al.</i> , 1989b; Rossol <i>et al.</i> , 1989
Chronic hepatitis B		Yes	Tolentino <i>et al.</i> , 1975; Abb <i>et al.</i> , 1985
Acute hepatitis		Yes	Levin and Hahn, 1982
Cytomegalovirus	Yes	Yes	Emodi and Just, 1974; Rhodes-Feuillette <i>et al.</i> , 1983
Autoimmune conditions			
Systemic lupus erythematosus	Yes	Yes	Preble <i>et al.</i> , 1982; Yee <i>et al.</i> , 1989
Juvenile arthritis	Yes		Arvin and Miller, 1984
Multiple sclerosis	Yes	Yes	Neighbor <i>et al.</i> , 1981 unpublished results
Neoplastic conditions			
Myelodysplastic syndrome		Yes	Okabe <i>et al.</i> , 1986
Chronic myelomonocytic leukemia		Yes	Okabe <i>et al.</i> , 1986
Hairy cell leukemia		Yes	Gutterman <i>et al.</i> , 1980
Hodgkin's lymphoma		Yes	Rassiga-Pidot and McIntyre, 1984
Non-Hodgkin's lymphoma		Yes	Ho <i>et al.</i> , 1988
Chronic lymphocytic leukemia		Yes	Lee <i>et al.</i> , 1984; Fernandez <i>et al.</i> , 1986
Other conditions			
Bell's palsy	Yes		Jonsson <i>et al.</i> , 1989
Pregnancy		Yes	unpublished results
Hemolytic uremic syndrome	Yes		Perez <i>et al.</i> , 1989

Sendai was not found to be correlated in the patient populations, with normal levels of IFN production in response to Sendai virus often seen in patients with grossly deficient NIPC function. Further, we have observed patients with normal Sendai ELISpot responses but very low frequency NIPC. For a portion of the most advanced patients, however, both the Sendai- and HSV-induced IFN responses can become deficient, suggesting that eventually the entire IFN- α leukocyte system becomes affected. Dysfunction of the monocytic IFN-producing cells in AIDS is supported by the studies of Gendelman *et al.* (1990, 1992), who have found a selective deficiency in HIV-infected monocytes to produce IFN in response to Sendai virus.

6.2. IFN RESPONSES IN OTHER HUMAN DISEASES

In addition to the well-studied IFN system in AIDS, aberrant IFN- α responses including elevated serum IFN and/or decreased *in vitro* IFN- α responses have been reported for a number of other human disease conditions, including infectious diseases, neoplastic or preneoplastic disease and autoimmune disease. A sampling of these IFN system alterations is shown in Table 3; this list is not intended to be comprehensive, but rather to represent examples of these aberrant responses that have been reported in the literature. Many of the studies have involved only very small numbers of patients and the magnitudes of the deficiencies vary greatly with the disease. For example, positive serum IFN responses may vary from as little as 8–16 IU/mL seen in patients with multiple sclerosis to as much as 300 IU/mL seen in some patients with advanced AIDS. For the *in vitro* studies, there is also considerable heterogeneity in IFN- α generation, with significant

deficiencies being either moderate (for example, in some of the leukemic patients) to severe (as seen in patients with advanced AIDS). Moreover, these studies have been performed with a variety of IFN-inducing agents and culturing techniques, so that it is not necessarily possible to make direct comparisons between these studies.

In addition, with the exception of the studies in PBMC from AIDS patients, the mechanisms behind the IFN deficiencies have usually not been addressed. For example, it is not known whether the transiently depressed IFN responses seen in patients with acute viral infections are explained by sequestering of NIPC at the sites of viral infection or whether they represent deficient production by cells that are temporarily blocked in their ability to produce IFN by functional exhaustion. In leukemic patients, it is possible that the deficient *in vitro* responses reflect not only suppression of the IFN-producing cells by the tumor (and, indeed, many immune parameters can be rendered abnormal in the leukemic patient), but also that the abundance of tumor cells may be masking a normal IFN response. In support of this possibility, Fernandez *et al.* (1986) separated PBMC from several patients with chronic lymphocytic leukemia on Percoll density gradients and were able to demonstrate good levels of IFN- α production among the light density cells in some of the patients. Thus, for these few patients, it can be hypothesized that either the IFN response was masked by the tumor cells or, alternatively, was being actively suppressed by the tumor cells. The IFN locus of chromosome 9 is deleted in whole or part in some acute leukemias (Colamonici *et al.*, 1991; Grandér *et al.*, 1992); however, it is doubtful that these deletions are affecting IFN production by NIPC.

An interesting series of observations have recently been made by F. Siegal and colleagues (personal communication) in studies of patients with hairy cell leukemia. Hairy cell leukemia has been found to respond well to *in vivo* IFN- α therapy and recombinant IFN- α is an approved therapeutic modality in the United States, as well as in many countries. Patients with hairy cell leukemia have been observed to have profoundly suppressed *in vitro* IFN production (Kurzrock *et al.*, 1991; F. Siegal *et al.*, personal communication). Of interest is the observation that when the hairy cell leukemia patients are treated with immunosuppressive drugs to combat the tumor cells, T cell numbers and functions are also suppressed and the patients become susceptible to opportunistic infections typical of those seen with AIDS patients (F. Siegal *et al.*, personal communication). These observations support the concept described in Section 6.1 for the AIDS patients that full susceptibility to opportunistic infections does not occur unless the patients have failure of both the CD4⁺ T cells and the NIPC, again emphasizing the partial functional redundancy of the adaptive and innate immune systems and the important physiological role of both the NIPC and the T cells.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

The IFNs are critical cytokines in the normal host, with the ability to modulate immune responses and exert antiviral activity. Their ability to arrest cell growth is important not only for offering possible therapeutic intervention in cases of uncontrolled growth, but also has more central implications for a role for IFN activity in regulating hematopoiesis. Although many cells in the body are able to produce IFNs under appropriate stimulus, the NIPC are the primary cells responsive to a variety of different stimuli and along with perhaps the monocytes have emerged as the most important IFN- α -producing populations in the peripheral blood. Although considerable progress has been made in the past several years regarding the characterization of the NIPC in the peripheral blood and to a lesser degree their regulation, there are many questions that remain unanswered about these cells. Foremost among these is the cellular identity of the NIPC; i.e. do these cells represent a unique lineage or do they belong to an already defined lineage of cells, such as the dendritic cell? The absence of the NIPC (and also of the NK cell) from hematopoietic development charts is striking. The cellular distribution of the NIPC is also unknown, since appropriate tissue studies have not been performed to determine whether the cells are able to move out of the periphery and into the tissues. Clearly, the most significant impairment to studies of the IFN- α system in human peripheral blood remains the inability to identify uniquely the NIPC. Although much progress has been made in the past several years regarding their enrichment and

characterization, these cells are still identified as much by what they are not (i.e. not T cells, B cells, NK cells, stem cells or macrophages) as by what they are (i.e. low density, HLA-DR⁺ cells). Continued effort is clearly indicated towards defining the lineage of these cells and towards finding unique cell surface determinants on the cells, which would allow for enumeration and selection of the NIPC. It is hoped that the ability to enrich for these extremely rare cells may enable the development of monoclonal antibodies specific for the NIPC; it should theoretically be much easier to produce and screen for appropriate monoclonal antibodies using enriched populations of cells rather than with populations containing less than 0.2% of the target population. Once monoclonal antibodies specific for NIPC are developed, rapid progress should ensue towards studying their tissue distribution and phenotypic vs functional presence in the peripheral blood and other organs of healthy and diseased individuals.

Development of an animal model for studying NIPC cells is also clearly warranted. Such an animal model, in, for example, mice, would allow characterization of the physiological role of these cells. With the exception of the NIPC-like cells described in the pig by Charley and Laude (1988), the presence of NIPC has not been clearly demonstrated in other species, although it would be presumed that analogous populations of cells should exist. Once a murine model for studying the NIPC is established, it will be interesting to create NIPC knock-out mice that lack the functional cell type. Creation of NIPC that are dysfunctional *in vivo* and *in vitro* might also be accomplished by transgenic techniques where a selectable promoter is attached to IFN genes, leading to suicide of cells expressing the IFN genes. Such animals could be used to determine the physiological role of the NIPC. For example, one could test the role of NIPC in viral infections or in NK cell lytic activity or in hematopoietic homeostasis by knocking out NIPC while retaining monocytic IFN-producing cells.

The mechanisms by which viruses and other interferogenic substances induce IFN in NIPC and other IFN-producing cells remain to be more fully defined. Since it is clear that viral replication is not required for induction of IFN in NIPC, it will be interesting to determine what class(es) of molecules are able to induce IFN generation in these cells. For example, as described in Section 4, viral glycoproteins have sometimes been implicated in IFN induction in NIPC. It will be interesting to determine whether viral glycoproteins are universal inducers of IFN- α or whether this is restricted to specific viral glycoproteins and to NIPC. Nothing is currently known about the receptors on the NIPC that might bind to the viral glycoprotein ligands. It will be important to determine whether the receptors are relatively nonspecific or whether they recognize specific viral glycoproteins and to determine whether binding of receptor and ligand is sufficient to transduce a signal to the NIPC to produce IFN.

It is well established that the NIPC, although very rare cells, are capable of producing large quantities of IFN upon appropriate stimulation. Whether these cells produce other cytokines has not been studied, nor have other potential physiological functions been evaluated, with the exception of their possible role as ACs for NK cell activity. One interesting question is the functional significance of the MHC Class II molecules constitutively expressed on the NIPC. The presence of Class II molecules on NIPC suggests that these cells might be important antigen-presenting cells in the immune system.

Finally, an understanding of immunopathogenesis that leads to destruction, redistribution or inactivation of NIPC, as seen in AIDS and other viral infections, neoplastic conditions and autoimmunity, needs to be developed. Ultimately, the goal of these studies will be to develop treatment modalities, which would be able to reverse the dysfunction of the IFN system in the patients and to help restore homeostatic balance.

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