Clinical Science (1995) 89, 511–519 (Printed in Great Britain)

Effects of different lipoproteins on the proliferative response of interleukin-2-activated T lymphocytes and large granular lymphocytes

Juan B. DE SANCTIS, Isaac BLANCA and Nicholas E. BIANCO

Institute of Immunology, Faculty of Medicine, Central University of Venezuela, Sabana Grande, Caracas, Venezuela

(Received 3 February/30 May 1995; accepted 15 June 1995)

1. T lymphocytes and large granular lymphocytes internalized chylomicrons, very low-density lipoprotein, low-density lipoprotein, high-density lipoprotein and acetyl modified low-density lipoprotein through different receptors as assessed by flow cytometry. The observed internalization ranged from 8% to 20%.

2. All lipoproteins induced proliferative responses in T lymphocytes and large granular lymphocytes at optimum concentrations (40 μg of protein/ml for all lipoproteins except high-density lipoprotein). Chylomicrons, very low-density lipoprotein and low-density lipoprotein increased T-lymphocyte proliferative response by fourfold while inducing respectively a seven-, nine- and sevenfold increment in large granular lymphocytes. Similarly, high-density lipoprotein and acetyl modified low-density lipoprotein respectively induced a nine- and sevenfold increment in T cells and a 17- and eightfold increment in large granular lymphocyte proliferative response.

3. Both cell types internalized more lipoprotein when they were stimulated with interleukin 2. Chylomicrons and low-density lipoprotein internalization was increased threefold and very low-density lipoprotein internalization twofold, while high-density lipoprotein internalization was unchanged in both cell types. Acetyl modified low-density lipoprotein internalization was fourfold higher in large granular lymphocytes only.

4. The proliferative response of interleukin-2 stimulated cells was different from that of unstimulated cells. Chylomicrons and very low-density lipoprotein induced a sixfold increment in T-cell proliferative response but only a fourfold increment in large granular lymphocytes. Low-density lipoprotein and acetyl modified low-density lipoprotein induced respectively a sevenfold and eightfold increment in T cells and a eightfold and threefold increment in large granular lymphocyte proliferative response. High-density lipoprotein did not affect T-lymphocyte proliferative response while inducing a twofold increase in large granular lymphocytes.

5. Lipoproteins are important in the proliferative response of unstimulated and interleukin-2-stimulated cells.

INTRODUCTION

Lipoproteins, chylomicrons (CMs), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) [1] have always been considered important for cell and tissue physiology although the deregulation of their metabolic control may be the cause of vascular pathologies such as atherosclerosis [2, 3].

Macrophages and T lymphocytes have been implicated in the formation of atheroma. The effects of oxidized LDL and lipoprotein remnants in the induction of foam cell formation have been documented [3–5] and the presence of different T-cell subpopulations in the lesion has been reported [3, 4]. Several receptors for different lipoproteins have been shown to be present in macrophages: the scavenger receptors [6], the α_2-macroglobulin receptor/LDL receptor-related protein (α_2-MR/LRP) [6], CD36 [7], the HDL receptors [8], VLDL receptors [9] and Fc receptors [10]. In T lymphocytes, only LDL receptors, which are up-regulated upon anti-CD3 activation [11], and receptors for modified LDL [1, 12] have been reported. However, little is known about the presence of lipoprotein receptors and their regulation in other lymphocytes.

Large granular lymphocytes (LGLs) ($CD3^-CD16^+CD56^+$) represent a subset of lymphocytes distinguishable from other lymphocytes by their morphology, phenotype and functional capacity to kill tumour cells or virally infected cells

Key words: chylomicrons, high-density lipoprotein, interleukin-2, large granular lymphocytes, low-density lipoprotein, T lymphocytes, very low-density lipoprotein.

Abbreviations: AcLDL, acetyl modified low-density lipoprotein, ADCC, antibody-dependent cell cytotoxicity; CM, chylomicron; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; FCS, fetal calf serum; HDL, high-density lipoprotein; IL-2, interleukin-2; LDL, low-density lipoprotein; LGL, large granular lymphocyte; LPL, lipoprotein lipase; α_2-MR/LRP, α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein; VLDL, very low-density lipoprotein.

Correspondence: Dr Juan B. De Sanctis, Institute of Immunology, Faculty of Medicine, Central University of Venezuela, Apartado 50109, Sabana Grande, Caracas 1050-A, Venezuela.

spontaneously [13, 14] and are one of the most important secretory cells of the immune system. In peripheral blood, LGLs account for about 5–15% of circulating lymphocytes, but in some organs, e.g. the liver, they represent up to 45% of tissue-infiltrating lymphocytes [14].

Interleukin-2 (IL-2) is a cytokine secreted by T lymphocytes and LGLs that has a range of effects on different cells of the immune system [15]. IL-2 is required for T-cell and LGL proliferative response [16] and enhances LGL tumoricidal activity, adhesion and locomotion, suggesting that IL-2 is a key cytokine for most LGL physiological responses [13, 14].

Although LGLs are not involved in the development of atherosclerosis, it has recently been shown that diet [17], lipid emulsions [18, 19], and LDL oxidized by polymorphonuclear leucocytes [20] alter LGL lytic efficiency against K562 and P815 cells. Furthermore, experiments with lovastatin (an inhibitor of cholesterol synthesis) *in vitro* have shown that it inhibits lymphocyte proliferative response and LGL cytotoxicity [21, 22]. This inhibition can be overcome by the addition of IL-2 [22, 23]. Thus, cholesterol synthesis and lipoprotein metabolism seem to affect LGL cytotoxic functions, but little is known about the proliferative response of these cells.

In this report, our aim was to study the internalization of the different lipoproteins in unstimulated and IL-2-stimulated T and large granular lymphocytes and to determine the effect of lipoproteins on the proliferative response of these cells.

MATERIALS AND METHODS

Chemicals

Recombinant human interleukin-2 (rhIL-2), fetal calf serum (FCS), L-glutamine, penicillin–streptomycin and RPMI-1640 medium were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) was purchased from Molecular Probes, OR, U.S.A.). Percoll and Ficoll-Hypaque were purchased from Pharmacia LKB (Uppsala, Sweden). [^{3}H]Thymidine was purchased from New England Nuclear (Boston, MA, U.S.A.). All other reagents were acquired from Sigma (St Louis, MO, U.S.A.).

Antibodies

Anti-Leu11c-PE (CD16) was purchased from Becton Dickinson (Mountain View, CA, U.S.A.); NKH-1 RD_1 (CD56), unlabelled CD3, CD3-FITC and CD14-FITC antibodies were purchased from Coulter Immunology (Hialeah, FL, U.S.A.).

Lipoprotein purification

All lipoproteins, CMs, VLDL, LDL and HDL were purified according to the method of Havel et al. [24]. Human plasma from fasted healthy donors was centrifuged at 114 000 *g* for 20 min at 16°C in the presence of inhibitors of lipoprotein oxidation [1 mmol/l butyl hydroxytoluene (BHT) 2 mmol/l reduced gluthatione, 5 mmol/l ascorbic acid and 5 mmol/l EDTA] to separate CMs from the plasma. CMs were subsequently washed using a discontinuous gradient (0.9% NaCl on the top and CM-KBr at the bottom) and centrifuged as described above. The infranatant after the removal of CMs was used to separate VLDL by centrifuging for 20 h at 114 000 *g* at 16°C and the purified fraction was washed twice with discontinuous gradients as described previously. LDL was purified adjusting the plasma density to 1.063 g/ml, centrifuged and cleaned as described for CM. The infranatant after the removal of CMs, VLDL and LDL was adjusted to a density of 1.2 g/ml to purify HDL then centrifuged and washed three times to remove contaminating albumin as described above. All lipoproteins were dialysed extensively against PBS–BHT–EDTA and before the assays against PBS alone. Protein contents were determined for each fraction using standard kits (Sigma). No oxidative intermediates were detected in the purified fractions using the thiobarbituric acid-reactive substrate assay for purified lipoproteins performed before cell proliferative studies and lipoprotein internalization studies [25]. No difference in migration was observed in the standard electrophoretic method in agarose gels (results not shown). The purified lipoproteins were endotoxin free as determined by the timed gel formation kit (Sigma).

Lipoprotein acetylation

LDL was acetylated using acetic anhydride as described previously by Basu et al. [26]. After acetylation was completed, acetyl modified LDL (AcLDL) was dialysed against PBS, filter sterilized and subsequently used for flow cytometric analysis and proliferative studies.. The electrophoretic mobility of this fraction was different from that of unmodified LDL (results not shown).

Labelling of lipoproteins with DiI

This procedure was selected since DiI is a non-toxic chromophore and does not change the physical properties of the lipoproteins [27]. Labelling of each lipoprotein with DiI was performed as described previously [27]. Briefly, sterile lipoproteins were adjusted to 2 mg/ml and then labelled with 200 μl of a 3 mg/ml solution of DiI dissolved in dimethylsulphoxide. The mixture was then added to 8 ml of lipoprotein-free plasma for 10 h at 37°C. Lipoprotein–DiI was centrifuged at 114 000 *g* for 20 minutes (chylomicrons) or for 18 h (VLDL, LDL and HDL) to eliminate the unbound chromophore. The supernatant, with the characteristic red colour, was dialysed against PBS, adusted to 2 mg/ml and

filter sterilized through a 0.45-μm Millipore filter. The labelling efficiency was determined from the emission of the chromophore in solution at 480 nm in a fluorimeter (Perkin-Elmer-Cetus, Norwalk, CT, U.S.A.), and was always close to 50% for all lipoproteins. In all the studies performed, the concentration of DiI was kept constant at 1 fluorescence unit of DiI per 1 μg of protein for all the lipoproteins studied.

Cell purification

Blood samples were taken from fasted normal healthy (normolipaemic) donors (blood bank of the Central University Hospital) with their written consent and with the approval of the ethical committee of the hospital. Human LGLs were separated by passage of the non-adherent mixed population of cells through nylon wool and subsequent centrifugation on Percoll gradients [28]. The cells isolated from the Percoll gradients were treated with anti-CD3 monoclonal antibody plus complement to deplete $CD3^+$ cells. The T cells obtained from the Percoll gradient were directly labelled for CD3 expression.

The purified fractions were assessed for CD16 and CD56 positivity and CD3 positivity respectively in an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, FL, U.S.A.). The purified LGL cell fraction contained >80% CD16, <2% CD3 and <1% MO2 positivity. The purified T ($CD3^+$) fraction was >90% positive. T lymphocytes and LGLs were then cultured for 18 h in RPMI-1640 medium supplemented with 0.5% BSA (fatty acid free) in the presence of different concentrations of IL-2 (0, 10, 100, 500 and 1000 i.u./ml). After the incubation, cell viability was higher than 90% as determined by trypan blue exclusion.

Flow cytometric studies

After 18 h incubation in RPMI–BSA the cells were washed in PBS, adjusted to 1×10^6 cells/ml, resuspended with different concentrations of lipoprotein–DiI (0, 8, 20, 40, 60, 80 and 100 μg/ml) in RPMI–BSA and incubated for 4 h at 37°C (maximum internalization observed, results not shown). After the incubation, the cells were extensively washed with PBS–0.5% BSA (fatty acid free) and the internalized lipoprotein was quantified by flow cytometry. The cytometer, previously calibrated with the fluorescence beads (Coulter Corporation), collected in the red photomultiplier (>570 nm) the signal generated from the emission of internalized DiI–lipoproteins excited at 488 nm with an argon laser. The positivity assessed with the flow cytometer corresponds to internalized lipoprotein. The unspecific binding was assessed by: (1) the positivity determined by adding lipoprotein–DiI to cells previously treated with 1 mmol/l EDTA and (2) the positivity determined for cells treated with lipoprotein–DiI and incubated for 4 h at 4°C.

Cell proliferative studies

After the 18-h incubation in RPMI–0.5% BSA fatty acid free and in the presence or absence of IL-2, the cells (>90% viable) were washed extensively with PBS, adjusted to $1 \times 10^5/100\,\mu$l in RPMI–BSA and added to 96-well plates that contained the different lipoproteins diluted with RPMI–BSA. The cells were cultured for 72 h, which was found to be the optimum period (data not shown). Eighteen hours before the end of the incubation, the cells were labelled with 1 μCi/ml [^{3}H]thymidine (prepared in RPMI–BSA) and the incorporation of [^{3}H]thymidine was measured in a beta-plate counter (LKB, Sweden). The viability at 72 h, calculated by trypan blue exclusion, was close to 80% for concentrations of lipoproteins up to 60 μg/ml. Cell viability decreases at lipoprotein concentrations higher than 60 μg/ml. HDL did not alter cell viability independently of the concentrations used.

In order to study the effect of lipoproteins in IL-2-activated T lymphocytes and LGLs, the cells were stimulated for 18 h with different concentrations of IL-2 (0, 10, 100 and 500 i.u./ml), washed and then cultured for 72 h with 20 μg/ml of the different lipoproteins.

Statistical analysis

The paired Student's t-test was used to analyse the results.

RESULTS

The maximum internalization for each lipoprotein assessed by flow cytometry in unstimulated and IL-2 stimulated T lymphocytes and LGLs is illustrated in Fig. 1. The observed internalization in unstimulated cells ranged between 8% and 20%, and was similar in LGLs and T lymphocytes, except for internalization of VLDL. VLDL internalization was higher in LGLs than in T cells (19 ± 3 versus 7 ± 3, $P < 0.05$, $n = 5$). IL-2 stimulation enhanced lipoprotein internalization in both cell types, and this effect was dependent on the concentration used. In T lymphocytes, CM internalization increased three- or fourfold when the cells were stimulated with 500 or 1000 i.u./ml IL-2 respectively. Similarly, threefold increment in VLDL and LDL internalization was observed when the cells were primed with 1000 i.u./ml IL-2. No major increase in AcLDL or HDL internalization was observed regardless of the concentration of IL-2 used. In LGLs, IL-2 activation increased the internalization of CMs threefold (500 i.u./ml) and fourfold (1000 i.u./ml), the internalization of VLDL twofold (100 and 500 i.u./ml) and 2.5-fold (1000 i.u./ml) and the internalization of LDL and AcLDL threefold (1000 i.u./ml). As

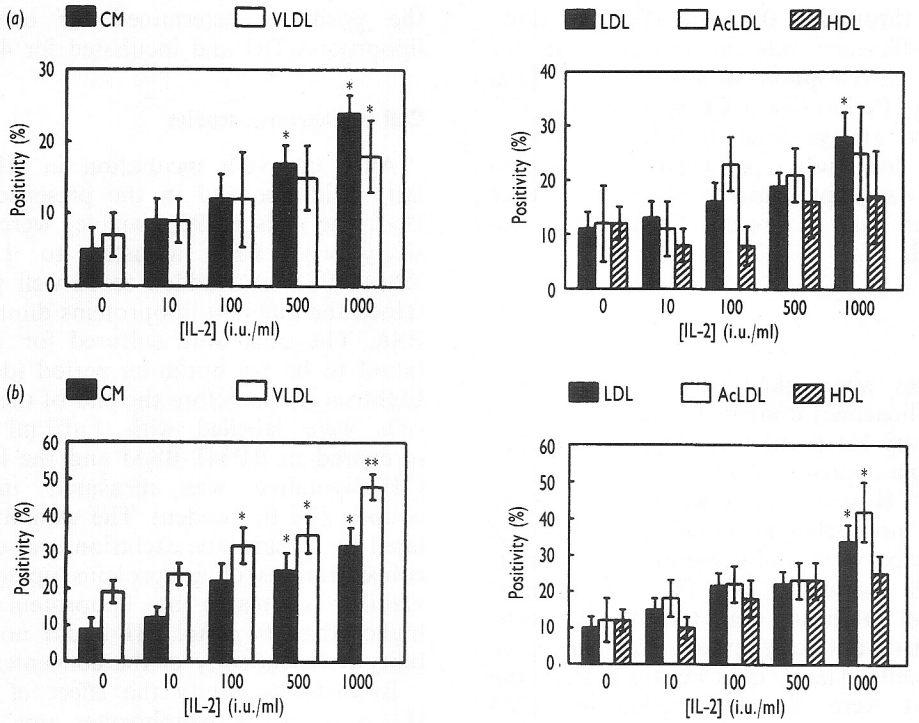

Fig. 1. Effect of interleukin 2 on the maximum internalization of different lipoproteins labelled with DiI in (*a*) T lymphocytes and (*b*) large granular lymphocytes. After 18 h incubation in the presence of different concentrations of IL-2 and in the absence of lipids (lipoproteins and free fatty acids), T lymphocytes and LGLs were washed and 1×10^6 cells were incubated with the different lipoproteins labelled with DiI as described in the Materials and methods section. The figure represents only the mean and the standard deviation of the maximum uptake for each lipoprotein (40 μg/ml), observed in five different donors performed in duplicate. Significant differences were observed when the positivity assessed in stimulated cells was compared with that in the unstimulated controls ($^*P < 0.05$, $^{**}P < 0.01$).

observed in T lymphocytes, HDL internalization was not increased in LGLs regardless of the concentration of IL-2 used.

Figs. 2 and 3 depict a typical flow cytometry quantification of CM and VLDL internalization in unstimulated T lymphocytes. T lymphocytes (Fig. 2) and LGLs (Fig. 3) stimulated with 1000 i.u./ml IL-2. Upon IL-2 stimulation, the positivity of all the events studied increased. CM internalization was increased in both cell types similarly. As shown in Fig. 1, VLDL internalization was higher in LGLs than in T lymphocytes and VLDL uptake was significantly higher in stimulated LGLs cells than in stimulated T lymphocytes ($48\% \pm 8\%$ versus $18\% \pm 4\%$, $P < 0.05$, $n = 5$).

Flow cytometry quantification of LDL, HDL and AcLDL internalization in T lymphocytes and LGL cells is shown in Figs. 4 and 5. In each case (*a*) represents unstimulated cells and (*b*) the expression in stimulated cells (1000 i.u./ml IL-2). Similar values for internalized lipoproteins were observed in unstimulated T lymphocytes and LGLs. Upon stimulation with IL-2, there is an increase in positivity in all cases, but this increase is significant only for LDL in both cell types and for AcLDL in LGLs, as reported in Fig. 1.

The effect of the different lipoproteins on the proliferative response of unstimulated T cells and LGLs is illustrated in Fig. 6. All lipoproteins, at optimal concentrations, induce a proliferative response in both cell types. CMs and VLDL concentrations higher than 40 μg/ml and LDL and AcLDL at concentrations higher than 60 μg/ml cause a reduction in cell proliferative response due to a decrease in cell viability. CMs and VLDL have similar effectiveness in the induction of the proliferative response in T lymphocytes and LGLs. However, in LGLs, the response is greater, with a seven- (CMs) and ninefold (VLDL) augmentation in the proliferative response as compared with fourfold increase induced by both lipoproteins in T lymphocytes. Similarly, the induction of the cell proliferative response by LDL, HDL and AcLDL is higher in LGLs than in T lymphocytes. LDL, AcLDL and HDL increased LGL proliferative response by seven-, eight- and 17-fold respectively, while LDL, AcLDL and HDL augmented T-lymphocyte proliferative response by four-, seven- and ninefold respectively.

In both cell types, an increment in the proliferative response was observed in IL-2-stimulated cells, as illustrated in Fig. 7. Lipoprotein enhanced cell proliferation in cells previously primed with IL-2, and these increments were higher in T lymphocytes than in LGLs, except for those caused by HDL. These results contrast with those reported in Fig. 6.

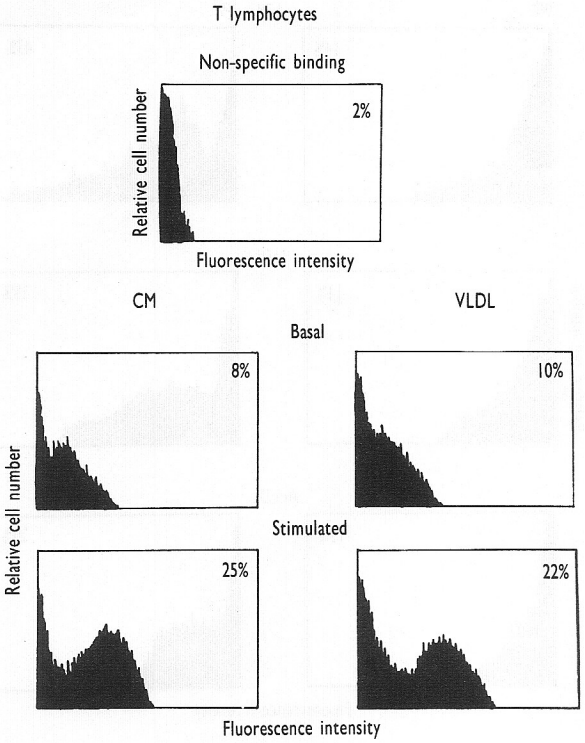

Fig. 2. Flow cytometry analysis of the internalization of CM–DiI and VLDL–DiI by T lymphocytes. Typical histograms of internalized CM–DiI and VLDL–DiI in non-stimulated (basal) and stimulated (1000 i.u./ml IL-2) T lymphocytes are shown. In the top part of the figure, the non-specific binding was assessed using cells incubated with lipoprotein–DiI in the presence of 1 mmol/l EDTA. This control is similar for any lipoprotein studied. The numbers on the right of each histogram represent the specific positivity observed.

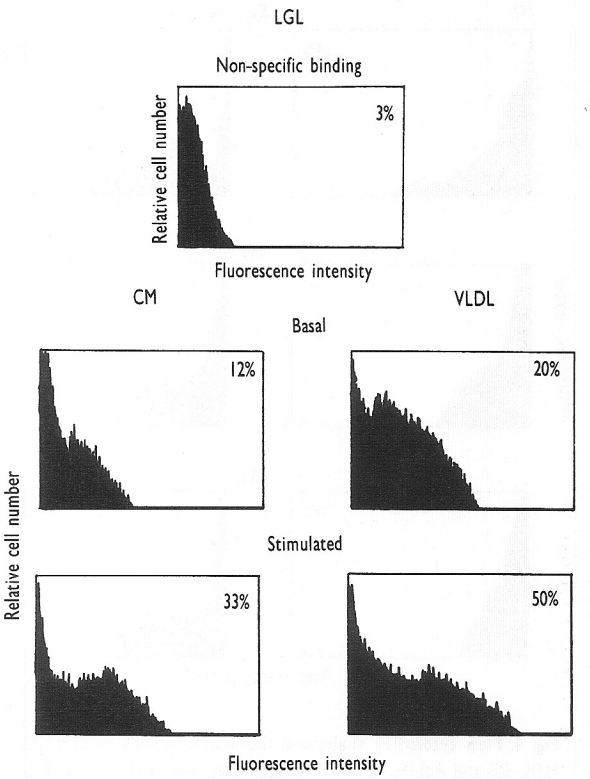

Fig. 3. Flow cytometry analysis of the internalization of CM and VLDL by LGLs. Typical histograms of internalized CM–DiI and VLDL–DiI in non-stimulated (basal) and stimulated (1000 i.u./ml IL-2) LGLs are shown. In the top part of the figure, the non-specific binding is illustrated as described in Fig. 2. The numbers on the right of each histogram represent the specific positivity observed.

The increase in cell proliferative response at doses higher than 100 i.u./ml IL-2 as compared with the unstimulated cells is significant in all cases ($P<0.00001$, $n=5$) except for HDL in T lymphocytes. Proliferative induction by CMs and VLDL (>100 i.u./ml IL-2) was increased sixfold in T cells compared with fourfold in LGLs. Similarly, LDL and AcLDL induced an eight- and sevenfold induction in T lymphocytes as compared with fourfold for both lipoproteins in LGLs. In addition, T-lymphocyte proliferative response does not reach a maximum when cells are stimulated with LDL and AcLDL, contrary to what was shown in Fig. 6. Finally, a twofold increase in proliferative response was induced in LGLs but not T lymphocytes by HDL.

DISCUSSION

Lipoprotein receptors are expressed in mononuclear cells when they are cultured in the absence of lipoproteins and fatty acids [1] and depend on complex post-transcriptional and post-translational mechanisms [1]. T lymphocytes and macrophages internalize lipoproteins through different mechanisms [1–12]. T lymphocytes have been shown to express LDL receptors, after their activation with mitogen or with anti-CD3 [11], and modified LDL receptors that induce their proliferation [12]. Macrophages express scavenger receptors, Fc receptors and the CD36 antigen, which preferentially bind modified LDL, the VLDL receptor and the α_2-macroglobulin receptor/LDL receptor-related protein (α2MR/LRP), which has been shown to be responsible for the internalization of CM and VLDL [6–10]. In other cells of the immune system only indirect evidence for lipoprotein receptors has been presented.

Dietary lipids [17], lipid emulsions [18, 19] and oxidized LDL [20] have been shown to alter spontaneous and antibody-dependent cell cytotoxicity (ADCC) of LGLs, suggesting that lipoprotein receptors may modulate LGL functions. Furthermore, lovastatin, an inhibitor of the key enzyme of the cholesterol pathway (hydroxyl methyl glutaryl CoA reductase), *in vitro*, is able to decrease the proliferative response due to mitogenic stimulus and suppress 50% of the cytotoxic response of LGLs [21, 22]. IL-2 reverses lovastatin inhibition, suggesting that IL-2 may modulate either cholesterol synthesis or cholesterol uptake in these cells. Despite its

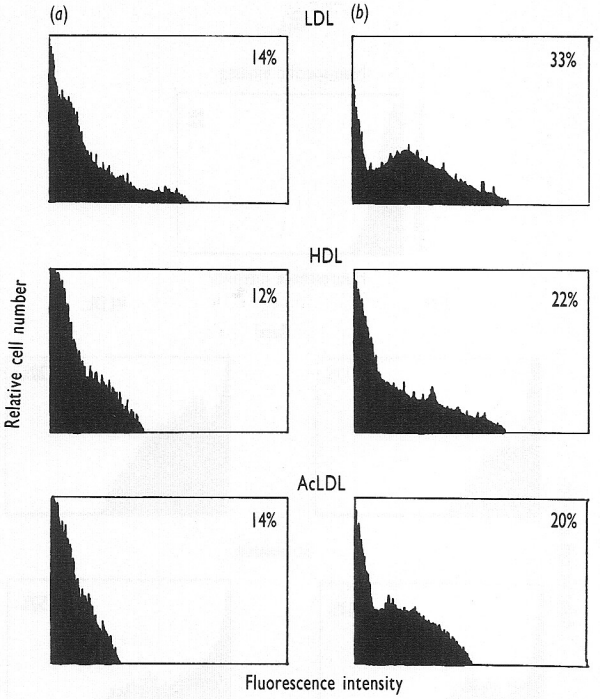

Fig. 4. Flow cytometry analysis of the internalization of LDL-DiI, HDL-DiI and AcLDL-DiI by T lymphocytes. Typical histograms of the internalized LDL-DiI and AcLDL-DiI in non-stimulated (*a*) (basal) and stimulated (*b*) (1000 i.u./ml IL-2) T lymphocytes are shown. (*a*) Positivity observed for internalized LDL-DiI, HDL-DiI and AcLDL-DiI in non-stimulated cells. (*b*) Uptake observed in T lymphocytes stimulated with 1000 i.u./ml IL-2. The numbers on the right of each histogram represent the specific positivity observed.

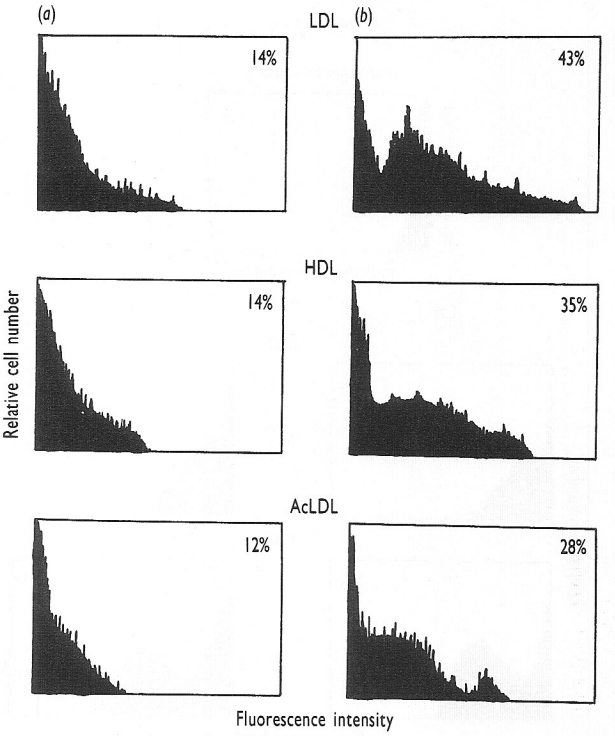

Fig. 5. Flow cytometry analysis of the uptake of LDL, HDL and AcLDL by large granular lymphocytes. Typical histograms of the internalized LDL-DiI and AcLDL-DiI in non-stimulated (*a*) (basal) and stimulated (*b*) (1000 i.u./ml IL-2) LGLs are shown. (*a*) Positivity observed for internalized LDL-DiI, HDL-DiI and AcLDL-DiI in nonstimulated cells. (*b*) Uptake of HDL and LDL observed in LGLs stimulated with 1000 i.u./ml IL-2 and of AcLDL in LGLs stimulated with 500 i.u./ml IL-2. The numbers on the right of each histogram represent the specific positivity observed.

importance, these reports did not assess the expression and function of lipoprotein receptors in LGLs.

In this report, we have shown that all lipoproteins are internalized by T cells and LGLs and that these lipoproteins are able to increase cell proliferative response at optimal concentrations. Both cell types are able to take up lipoproteins, although the amount of internalized lipoprotein is not very large. Only the internalization of VLDL was higher in LGLs than in T lymphocytes. The internalization of these lipoproteins induces an increase in cell proliferative responses that may range from fourfold up to 17-fold induction (HDL) in both cell types. The proliferative response of unstimulated LGLs is higher than the response observed in T lymphocytes. As reported previously [1–5], most lipoproteins (CM, VLDL, LDL and AcLDL) at concentrations higher than 60 μg/ml decreased cell viability, probably because of lipoprotein toxicity.

IL-2 was shown to increase the internalization of all lipoproteins except HDL in both cell types. There was a significant increase in the internalization of CMs, VLDL and LDL in both cell types and of AcLDL only in LGLs upon IL-2 stimulation. This enhancement in the internalization of CMs, VLDL, LDL and AcLDL did not resemble the proliferative response observed in both cell types. IL-2-stimulated T lymphocytes internalize less CMs, VLDL, LDL and AcLDL than IL-2-stimulated LGLs; however, T cells had higher proliferative responses than LGLs. These results differ from the values obtained with unstimulated cells. These differences suggest that IL-2, in both cell types, may facilitate lipoprotein internalization.

In contrast to the other lipoproteins, there were no significant increments in HDL internalization in both cell types upon IL-2 stimulation. In macrophages, it has been shown that HDL receptors are present [8] and that internalization of HDL does not involve lysosomal degradation [29]. A similar mechanism may be postulated for T lymphocytes and LGLs. Furthermore, adding HDL to IL-2-stimulated T cells did not enhance their proliferative response significantly, but induced a twofold increase in LGL proliferative response. These results are opposite to the effects of HDL in unstimulated T lymphocytes and LGL, which are similar to those reported by Jürguens et al. [30] in a whole lymphocyte population.

Various members of the LDL receptor family (LDL receptor, VLDL receptor, LDL receptor related protein and gp330) have been reported in several cell lines [6] and their expression has been

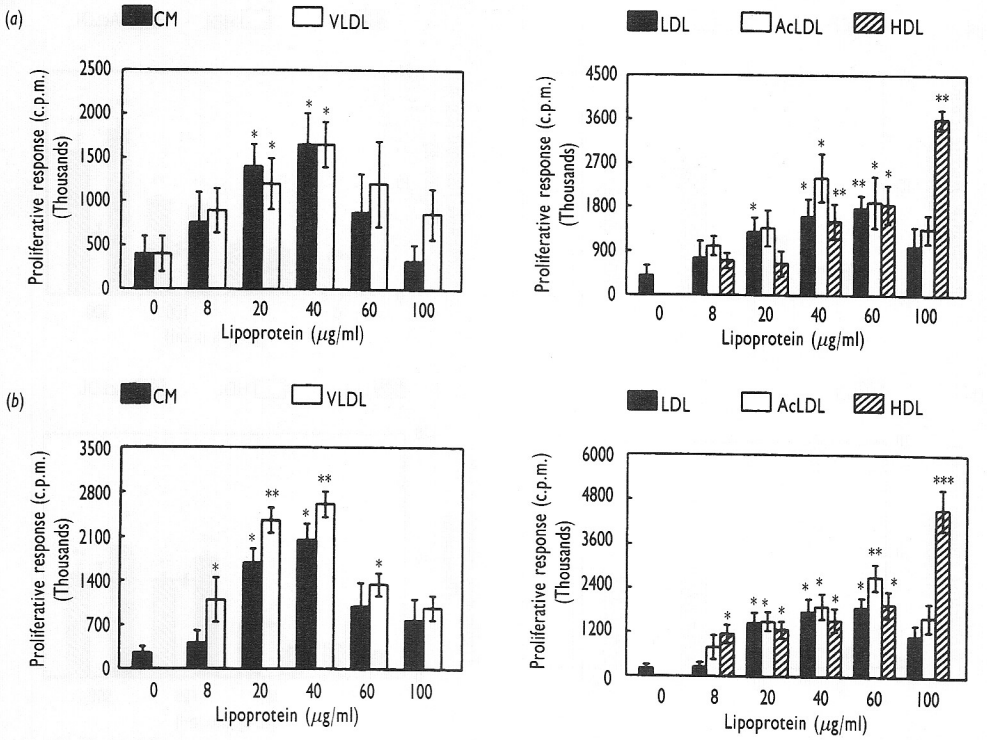

Fig. 6. Effect of lipoproteins on the proliferative response of non-stimulated (*a*) T lymphocytes and (*b*) LGLs. After 18 h incubation without stimulus in medium with BSA in the absence of fatty acids and lipoproteins, the cells were washed and 1×10^5 cells/100 μl were incubated with the different lipoproteins in 96-well plates for 72 h as described in the Materials and methods section. The results represent the mean and the standard deviation of five different individuals performed in triplicate. Significant increments in the proliferative response were observed in both cell types ($^*P < 0.05$ and $^{**}P < 0.01$) upon the addition of lipoprotein as compared with the controls to which no lipoprotein was added.

implicated in the internalization of different lipoproteins (LDL, CMs and VLDL). The internalization of these lipoproteins is dependent either on the specific receptor (LDL receptor, VLDL receptor) or on the formation of a complex with proteoglycans and/or lipoprotein lipase (LPL) (α2MR/LRP, gp330) [6]. LDL receptors have been shown to be present in T lymphocytes [1, 3, 12, 31, 32] and LGLs [33], but there are no reports on the expression, function and regulation of other members of the LDL receptor family in these cells.

We have recently shown that LGLs but not T or B lymphocytes express LPL on their surface [23]. LPL has been shown to bind CMs and VLDL, and this selective binding induces the internalization of the complex through α_2-MR/LRP in macrophages [34–38]. A tentative explanation for the higher internalization of CMs and VLDL by unstimulated LGLs is that LPL is released from the surface of LGLs as a result of its affinity for triglycerides and the complex LPL–CM or LPL–VLDL is internalized through α_2-MR/LRP. In addition, IL-2 induces LPL release from the LGL cell surface [23], facilitating the formation of the complex, which would then be internalized by α_2MR/LRP, this may account for the difference observed.

Despite the fact that LDL induces identical responses in both cell types (although the magnitude of response differs), AcLDL did not seem to induce the same responses in IL-2-activated cells. In T lymphocytes, IL-2 did not increase AcLDL internalization, but addition of AcLDL to IL-2-stimulated cells produced an augmentation in the proliferative response similar to LDL. In LGLs, IL-2 induced an increase in the internalization of AcLDL that paralleled the increase in proliferative response. The proliferative response of T lymphocytes is, however, twofold higher than that of LGLs. The internalization of AcLDL in both cell types may be dependent on scavenger receptors, or other receptors, such as Fc receptors, that might induce different cellular responses. Previous reports have shown that oxLDL induces an increase in T-lymphocyte proliferative response [12] and inhibits LGL antibody-dependent cell cytotoxicity [20]. The scavenger receptors have been shown to be down-regulated by lipopolysaccharide, transforming growth factor beta and interferon gamma [6]; however, little is known about the effects of IL-2 despite its importance in various cell functions [15].

The presence of different lipoprotein receptors on the two cell types suggests an interaction between lipoprotein metabolism and proliferative response. This proliferative response induced by lipoproteins does not involve previous activation of the cells. Nevertheless, the addition of lipoproteins to IL-2-activated cells, except for HDL in T lymphocytes, potentiates the cell proliferative response. These

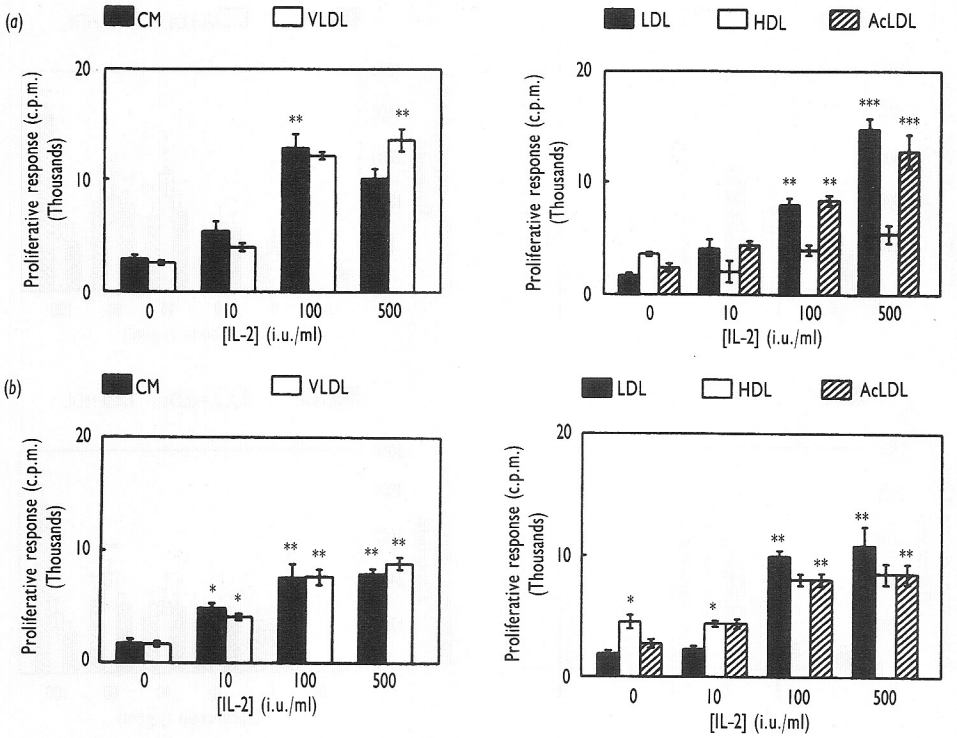

Fig. 7. Effect of lipoproteins on the proliferative response of IL-2-stimulated (*a*) T lymphocytes and (*b*) LGLs. After 18 h incubation in the presence of stimulus in medium with BSA in the absence of lipids, the cells were washed and 1×10^5 cells/100 μl were incubated with the different lipoproteins in 96-well plates for 72 h as described in the Materials and methods section. The results represent the mean and the standard deviation of five different individuals performed in triplicate. Significant increments in the proliferative response were observed in both cell types ($^*P < 0.05$ and $^{**}P < 0.01$, $^{***}P < 0.005$) as compared with the controls to which no lipoprotein was added.

results suggest the involvement binding of lipoproteins to their receptors stimulates cell signals that eventually would lead to the generation of cytokines important for cell proliferation. IL-2 is important for T-lymphocyte and LGL proliferative response [15, 16], but both cell types require a co-stimulatory signal for optimal proliferative response. These co-stimulatory signals may be partly generated by lipoprotein addition to cell cultures.

In summary, we have shown that T lymphocytes and LGLs internalize the different types of lipoproteins with distinct avidities. The internalization may be dependent on different receptors: (a) receptors of the family of LDL receptors (LDL receptor, VLDL receptor and α_2-MR/LRP receptor), (b) scavenger receptors or Fc receptors and (c) HDL receptors. These receptors seem to activate cells' proliferative response through different mechanisms. The biochemical basis of these effects is as yet unknown, but its elucidation will provide more insight into the importance of triglyceride, phospholipid and cholesterol homeostasis in the physiological responses of T cells and LGLs and their likely role in atherosclerosis.

ACKNOWLEDGMENTS

These studies were supported by the CONICIT (Consejo Nacional de Investigaciones Científicas y Tecnológicas, Venezuela) Grant S1-2516 and by the GENIC program. We thank Drs José Corado, Lorea Baroja and Jenny Garmendia for useful discussions and helpful comments.

REFERENCES

1. Traill KN, Huber LA, Wick G, Jürgens G. Lipoprotein interactions with T cells, an update. Immunol Today 1990; **11:** 411–17.
2. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: implication for cholesterol deposition in atherosclerosis. Annu Rev Biochem 1983; **52:** 223–61.
3. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature (London) 1993; **326:** 801–9.
4. Libby O, Hanson GK. Involvement of the immune system in human atherosclerosis: current knowledge and unanswered questions. Lab Invest 1991; **64:** 5–15.
5. Parthasarathy S, Rankin SM. Role of oxidized low density lipoproteins in atherosclerosis. Prog Lipid Res 1992; **31:** 127–43.
6. Kriger M, Hertz J. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor related protein (LRP). Annu Rev Biochem 1994; **63:** 601–37.
7. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Potter AA. CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem 1993; **268:** 11811–16.
8. Alam R, Yatsu RM, Tsui L, Alam S. Receptor-mediated uptake and 'retroendocytosis' of high-density lipoproteins by cholesterol-loaded human monocyte-derived macrophages: possible role in enhancing reverse cholesterol transport. Biochim Biophys Acta 1989; **1004:** 292–9.
9. Takahashi S, Kawarabayasi Y, Nakai T, Sakai J, Yamamoto T. Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. Proc Natl Acad Sci USA 1992; **89:** 9252–6.

10. Stanton LW, White RT, Bryant CM, Protter AA, Endemann G. A macrophage Fc receptor for IgG is also a receptor for oxidized low density lipoprotein. J Biol Chem 1992; **267:** 22446–51.
11. Suzuki K, Hara M, Kitani A, et al. Augmentation of LDL receptor activities on lymphocytes by interleukin 2 and anti CD3 antibody: a flow cytometric analysis. Biochim Biophys Acta 1990; **1042:** 352–63.
12. Frostergård J, Wu R, Giscombe R, Holm G, Lefvert AK, Nilsson AK. Induction of T cell activation by oxidized low density lipoprotein. Arterioscler Thromb 1992; **12:** 461–7.
13. Trinchieri G. The biology of NK cells. Adv Immunol 1989; **47:** 187–376.
14. Whiteside TL, Herberman RB. Role of human killer cells in health and disease. Clin Diagn Lab Immunol 1994; **1:** 125–33.
15. Hawkins MJ. Interleukin 2 antitumor and effector cell responses. Semin Oncol 1993; **20:** 52–9.
16. Robertson MJ, Manley TJ, Donahue H, Levine H, Ritz J. Costimulatory signals are required for optimal proliferation of human natural killer cells. J Immunol 1993; **150:** 1705–14.
17. Yaqoob P, Newsholme EA, Calder PC. Inhibition of natural killer cell activity by dietary lipids. Immunol Lett 1994; **41:** 241–6.
18. Kurzer M, Tice D, Meguid MM, Reinitz ER. Natural killer cell activity in rats infused with Intralipid^{R}. J Clin Lab Immunol 1989; **29:** 33–5.
19. Sedman PC, Somers SS, Ramsden CW, Brennan TG, Guillou PJ. Effects of different lipid emulsion on lymphocyte function during total parenteral nutrition. Br J Surg 1991; **78:** 1396–1400.
20. Tanabe F, Sato A, Ito M, Ishida E, Ogata M, Shigeta S. Low density lipoprotein oxidized by polymorphonuclear leukocytes inhibits natural killer cell activity. J Leuk Biol 1988; **43:** 204–10.
21. Cutts JL, Bankhurst AD. Suppression of lymphoid cell functions *in vitro* by inhibition of 3-hydroxy-3 methylglutaryl Coenzyme A reductase by lovastatin. Int J Immunopharmacol 1989; **11:** 863–9.
22. Cutts JL, Bankhurst AD. Reversal of lovastatin mediated inhibition of natural killer cell cytotoxicity by interleukin 2. J Cell Physiol 1990; **145:** 244–52.
23. De Sanctis JB, Blanca I, Radzioch D, Bianco NE. Lipoprotein lipase expression in natural killer cells and its role in their cytotoxic activity. Immunology 1994; **83:** 232–9.
24. Havel RJ, Eder HH, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955; **34:** 1345–53.
25. El-Saadani M, Esterbauer H, El-Sayed M, Goher M, Nassar AY, Jürgens G. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. J Lipid Res 1989; **30:** 627–30.
26. Basu SK, Goldstein JL, Anderson RGW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc Natl Acad Sci USA 1976; **73:** 3178–82.
27. Stephan ZF, Yurachek EC. Rapid fluorometric assay of LDL receptor activity by Dil-labeled LDL. J Lipid Res 1993; **34:** 325–9.
28. Timonen T, Ortaldo JR, Herbermann R. Characteristics of human large granular lymphocytes and relationship to natural killer cells and K cells. J Exp Med 1981; **153:** 569–79.
29. Rahim ATMA, Miyazaki A, Morino Y, Horiuchi S. Biochemical demonstration of endocytosis and subsequent resecretion of high-density lipoprotein by rat peritoneal macrophages. Biochim Biophys Acta 1991; **1082:** 195–207.
30. Jürgens G, Xu Q-b, Huber LA, Böck G, Howanietz H, Wick G, Traill KN. Promotion of lymphocyte growth by high density lipoproteins (HDL). J Biol Chem 1989; **264:** 8549–56.
31. Cuthbert JA, Russell DW, Lipsky PE. Regulation of low density lipoprotein receptor gene expression in human lymphocytes. J Biol Chem 1989; **264:** 1298–1304.
32. Cuthbert JA, Lipsky PE. Mitogenic stimulation alters the regulation of LDL receptor gene expression in human lymphocytes. J Lipid Res 1990; **31:** 2067–78.
33. De Sanctis JB, Blanca I, Radzioch D, Bianco NE. Low density lipoprotein receptor is expressed in NK cells and affects proliferative and cytotoxic activity of these cells. J Leuk Biol 1994; **56:** 34, A103.
34. Hussain M, Maxfield FR, Más-Oliva J, et al. Clearance of chylomicron remnant by the low density lipoprotein receptor mediated protein/α_2 macroglobulin receptor. J Biol Chem 1991; **266:** 13936–40.
35. Beisiegel U, Weber W, Bengtsson-Olivecrona G. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. Proc Natl Acad Sci USA 1991; **88:** 8342–6.
36. Chappell DA, Fry GL, Waknitz MA, et al. Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor *in vitro*. A process facilitated by cell surface proteoglycans. J Biol Chem 1993; **268:** 14168–75.
37. Williams SE, Inoue I, Tran H, et al. The carboxy-terminal domain of lipoprotein lipase binds to the low density lipoprotein receptor-related protein α_2 macroglobulin receptor (LRP) and mediates binding of normal very low density lipoprotein to LRP. J Biol Chem 1994; **269:** 8653–9.
38. Mulder M, Lmbardi P, Jansen H, van Berkel TJC, Frants RR, Havekes LM. Low density lipoprotein receptor internalizes low density and very low density lipoproteins that are bound to heparan sulfate proteoglycans via lipoprotein lipase. J Biol Chem 1993; **268:** 9369–75.

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 1995, p. 404–407
1071-412X/95/$04.00+0

Vol 2, No. 4

Decreased T-Cell Proliferative Response to Common Environmental Antigens Could Be an Indicator of Early Human Immunodeficiency Virus-Mediated Lymphocyte Lesions

P. TASSINARI, L. DEIBIS, I. BLANCA, N. E. BIANCO, AND G. ECHEVERRÍA DE PÉREZ*

Instituto de Inmunología, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela

Received 17 October 1994/Returned for modification 13 January 1995/Accepted 13 March 1995

To evaluate $CD4^+/CD29^+$ cells and their responses to different antigens in polar stages of human immunodeficiency virus (HIV) infection, we studied 26 HIV-seropositive carriers (SPCs) and 15 patients with AIDS simultaneously with 20 healthy volunteers (HVs) and 10 seronegative homosexual and bisexual men (SNH). CD3, CD4, CD29, and CD45RA phenotypes were analyzed by two-color flow cytometry. Significant depletion of $CD4^+$ T cells and both memory ($CD4^+/CD29^+$) and naive ($CD4^+/CD45RA^+$) T-cell subsets was found among SPCs and AIDS patients compared with the numbers of such cells in the HV and SNH groups. Responses to optimal doses of *Candida albicans*, streptokinase, and tetanus toxoid were explored in peripheral blood mononuclear cells and $CD4^+$- and $CD4^+/CD29^+$-enriched cell populations. In SPCs, the response to *C. albicans* in peripheral blood mononuclear cells showed a statistically significant diminution compared with the response of HVs (15,308 versus 35,951 cpm). In addition, a significantly reduced response to streptokinase was evident only when cell preparations were $CD4^+/CD29^+$ enriched (3,048 versus 10,367 cpm). Furthermore, the SPC group comprised seven responders to at least one antigen and seven nonresponders to any of the selected specific antigens. Absence of a response in these latter patients was independent of the absolute counts of memory and naive T-cell populations. The response to tetanus toxoid, although diminished in SPCs, was not significantly different from that in controls. Our results suggest that defective responses to common environmental antigens, unrelated to the absolute number of $CD4^+/CD29^+$ cells, is probably an early indicator of an HIV-induced lymphocyte lesion.

The human immunodeficiency virus (HIV) is related to a wide spectrum of immunological abnormalities in humans (2, 8). $CD4^+$ lymphocytes are particularly vulnerable to HIV, which in fact induces selective dysfunction and destruction of this population. Recently, investigative efforts have been focused on the interaction between HIV and $CD4^+$ evolutionary subsets, naive ($CD4^+/CD45RA^+$) and memory ($CD4^+/CD29^+$) cells. These subsets display different functional capabilities; naive cells respond to mitogens and autologous lymphocyte stimuli but do not proliferate in response to soluble antigens; in contrast, memory cells recognize recall antigens (14, 15). Altered proliferative responses to soluble antigens, alloantigens, and mitogens, plus depletion of both naive and memory cell subsets, have been reported in patients with HIV infection (6, 7, 10, 11, 13, 18).

We assessed the number and functional status of $CD4^+$ and $CD4^+/CD29^+$ memory cell subsets in asymptomatic HIV-infected individuals with the aim of gaining further insight into the natural history of the immunopathology of the HIV-$CD4^+$ cell subset interaction.

MATERIALS AND METHODS

Patients and controls. Twenty-six HIV-seropositive carriers (SPCs) and 15 male patients with AIDS were investigated. Ten homosexual or bisexual men were included as a high-risk seronegative reference group (SNHs); in addition, 20 healthy heterosexual volunteers (HVs) were also evaluated as controls.

Antibodies against HIV. Antibodies to HIV were assessed in all patients and controls by enzyme-linked immunosorbent assays (Abbott Recombinant HIV-1 EIA; Abbott Laboratories; Vironostika anti-HTLV-III, Organon Teknika, Boxtel, Holland), and the result was confirmed by Western blot (immunoblot) analysis (HIV-1 Western blot kit, Organon Teknika).

Phenotype analysis. Cell surface markers were determined by single- and dual-color flow cytometry (EPICS-753; Coulter Corporation, Hialeah Fla.). Fluorescein isothiocyanate- or RD1-tagged monoclonal antibodies against CD3, CD4, CD8, CD29, and CD45RA cells (Coulter Corporation) were added to EDTA-treated peripheral blood; after 10 min of incubation, the samples were processed in a Coulter Q-Prep Immunology Work Station (Coulter Corporation); total leukocytes and differential counts were assessed in the same blood sample.

Cell preparation. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood by centrifugation over Ficoll-Hypaque gradients (3). Adherent cells were removed by incubation in plastic petri dishes (Falcon Labware, Becton Dickinson, Lincoln Park, N.J.); 10 ml of cell suspension (2×10^6 cells per ml) was used. Nonadherent cells were carefully removed, washed, and resuspended in RPMI–20% normal human serum (NHS) for a second cycle on plastic petri dishes; viability determined by trypan blue exclusion was 99%, and the proportion of peroxidase-positive cells ranged from 1 to 3%. Nonadherent cells were B cell depleted by passage through nylon columns (12); briefly, 12-ml syringes were packed with 1 g of nylon wool (Robbins Scientific, Mountain View, Calif.), autoclaved, washed with RPMI–10% NHS, and warmed at 37°C. The cell suspension was incubated in the column for 1 h at 37°C, and nonadherent cells were recovered by elution with prewarmed RPMI–10% NHS.

A panning technique (19) was used to obtain a $CD4^+/CD29^+$-enriched cell preparation by negative selection; briefly, 10×10^6 PBMCs depleted of B and adherent cells were incubated with 50 μl of anti-$CD8^+$ monoclonal antibody for 30 min before addition of the cells to plastic petri dishes (15 by 100 mm; Falcon Labware, Becton Dickinson) previously coated with goat anti-mouse immunoglobulin G (Atlantic Antibodies, Scarbourough, Maine). The dishes were then incubated at 4°C for 1 h, after which the nonadherent cells were carefully collected. Cells were washed and adjusted, and the procedure was repeated with 2H4 monoclonal antibody in order to finally obtain a $CD4^+/CD29^+$-enriched cell subpopulation; flow cytometric analysis showed more than 90% $CD4^+/CD29^+$ cells and fewer than 3% $CD8^+$ and $CD4^+/CD45RA^+$ cells.

Cell cultures. A total of 10^5 cells (PBMCs or $CD4^+$ or $CD4^+/CD29^+$ cells) were cultured with preestablished optimal doses of the following soluble antigens: tetanus toxoid (TT; 2.5 Lf/ml), kindly supplied by the Instituto Nacional de

* Corresponding author. Mailing address: Institute of Immunology, c/o Aerocav No. 1216, P.O. Box 025304, Miami, FL 33102-5304. Phone and Fax: (582) 672-0371.

TABLE 1. Phenotypic analysis of T cells and T-cell subsets in patients and controls

Cell	Amt (mm^3 [%]) of fluorescent cells in the following subjects[a]:			
	HVs (n = 20)	SNHs (n = 10)	SPCs (n = 26)	AIDS patients (n = 15)
Leukocytes	8,358 ± 2,745	8,272 ± 2,569	7,354 ± 2,949	5,610 ± 1,600[b]
Lymphocytes	3,164 ± 978 (39 ± 10)	3,352 ± 1,012 (42 ± 12)	2,676 ± 1,067 (38 ± 11)	786 ± 818[c] (31 ± 12)
$CD3^+$	2,237 ± 612 (72 ± 7)	2,586 ± 990 (75 ± 10)	2,030 ± 1,064 (74 ± 16)	1,245 ± 753[c] (68 ± 18)
$CD29^+$	2,349 ± 821 (74 ± 12)	2,919 ± 870 (87 ± 4[b])	1,924 ± 973 (71 ± 17)	1,287 ± 714[c] (73 ± 19)
$CD45RA^+$	2,243 ± 766 (72 ± 13)	2,722 ± 880 (81 ± 6[b])	1,927 ± 871 (72 ± 10)	1,138 ± 647[c] (69 ± 12)
$CD3^+/CD29^+$	1,772 ± 646 (53 ± 12)	2,210 ± 828[b] (64 ± 10[b])	1,430 ± 1,007 (51 ± 24)	893 ± 648[b] (49 ± 22)
$CD3^+/CD45RA^+$	1,538 ± 625 (50 ± 15)	2,219 ± 895[b] (62 ± 14[b])	1,333 ± 888 (47 ± 18)	789 ± 547[c] (43 ± 18)
$CD4^+$	1,335 ± 500 (43 ± 8)	1,425 ± 754 (40 ± 11)	679 ± 358[b] (25 ± 7[b])	183 ± 194[c] (10 ± 10[c])
$CD4^+/CD29^+$	998 ± 377 (31 ± 9)	1,223 ± 604 (33 ± 9)	455 ± 290[c] (16 ± 7[c])	104 ± 112[c] (5 ± 4[c])
$CD4^+/CD45RA^+$	759 ± 426 (25 ± 11)	986 ± 594 (27 ± 10)	406 ± 302[c] (14 ± 7[c])	104 ± 90[c] (5 ± 4[c])
$CD8^+$	899 ± 326 (29 ± 8)	1,126 ± 456 (34 ± 9)	1,366 ± 833[b] (49 ± 15[c])	971 ± 625 (54 ± 18)
$CD4^+/CD8^+$	1.62 ± 0.6	1.25 ± 0.6	0.6 ± 0.3[c]	0.27 ± 0.47[c]

[a] Results are expressed as the arithmetic mean ± standard deviation of the absolute amount (cubic millimeters) and percentages of fluorescent cells by flow cytometry.
[b] $P < 0.005$ when compared with HVs.
[c] $P < 0.001$ when compared with HVs.

Higiene, Caracas, Venezuela; *Candida albicans* (75 μg/ml), kindly donated by the Instituto de Biomedicina, Caracas, Venezuela; and streptokinase (SK; 62.5 IU/ml; Kabikinase; Kabivitrom, Stockholm, Sweden). Ten percent autologous adherent cells were added to $CD4^+$- and $CD4^+/CD29^+$-enriched subpopulations. Cultures were carried out in flat-bottom microtiter plates (Falcon Labware, Becton Dickinson); each well contained a final volume of 0.2 ml of RPMI 1640 medium supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated NHS. Triplicate cultures of each combination were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 7 days, pulsed with 1 μCi of tritiated thymidine 18 h before harvesting, and counted in a Betaplate Liquid Scintillation Counter (Wallac Oy, Turku, Finland). The results were expressed as the mean counts per minute of triplicate cultures.

Statistical analysis. The Student t test for two independent samples of unequal size was used, and P values were determined.

RESULTS

Patients and controls. Patients included 41 HIV-infected male individuals (ages, 19 to 61 years; mean age, 33 ± 9 years) whose HIV infections were classified according to the criteria of the Centers for Disease Control and Prevention (CDC) (5); 26 patients were asymptomatic (CDC group II) SPCs and 15 had AIDS (CDC group IV). All patients were confirmed to have antibodies to HIV by Western blotting.

Control individuals were also males (ages, 24 to 39 years; mean age, 31 ± 4 years); 10 were SNHs and 20 were HVs. All control subjects were screened for HIV antibodies, with negative results.

T-cell subpopulations. Results of phenotypic analysis of leukocytes, lymphocytes, T cells, and T-cell subpopulations in controls and patients are provided in Table 1. Both HVs and SNHs exhibited similar cell subpopulations with the exception of $CD3^+/CD29^+$ and $CD3^+/CD45RA^+$ T cells, which were present in significantly higher numbers in SNH individuals ($P < 0.005$).

The numbers of leukocytes, lymphocytes, and CD3 cells in SPCs were comparable to those in HVs. However, the numbers of $CD4^+$ cells (single-color flow cytometry) and both $CD4^+/CD29^+$ and $CD4^+/CD45RA^+$ (dual-color flow cytometry) appeared significantly reduced compared with the numbers of such cells in both HVs and SNHs. As expected, all lymphocyte subpopulations studied in AIDS patients were significantly diminished (Table 1).

Proliferative response to soluble antigens. Functional lymphocyte studies were performed in 11 controls, 10 SNHs, 14 SPCs, and 6 AIDS patients. When PBMC proliferation was evaluated, SPCs showed diminished responses to *C. albicans*, TT, and SK, reaching significance only in the case of the *C. albicans* antigen. A similar proliferation pattern was found in the responses of $CD3^+/CD29^+$-enriched cells from SPCs. However, when the responses from $CD4^+/CD29^+$ cells were studied, not only was there a diminished proliferation in response to the three antigens but significance was also again detected in the case of the *C. albicans* and SK antigens. As expected, in all instances, the responses among patients with AIDS were basically absent (Table 2).

When analyzing the proliferative responses among the SPCs, a remarkable individual variability was observed; this allowed us to divide the whole group into responders (R), when proliferative responses to one or more antigens were found, and nonresponders (NR), when the absence of a response to the three antigens was detected (Table 3). Proliferation among the R group was similar to that among the controls (HVs). Furthermore, to assess whether quantitative depletion of $CD4^+$ cells and/or memory cells ($CD4^+/CD29^+$) was responsible for the impaired responses, phenotypic analysis of cells from the R and NR groups was done. Table 4 shows no differences among T-cell subsets in the R and NR groups. Moreover, the numbers of both $CD4^+$ and $CD4^+/CD29^+$ cells were significantly depleted in both groups compared with the numbers in the HV group.

DISCUSSION

Depletion and functional abnormalities of CD4 lymphocytes are probably the most significant immunopathological lesions induced by HIV infection in humans (7, 11). In recent years, the emphasis has been placed on trying to further dissect the HIV-CD4 cell subset interaction to determine the natural history of the involvement of both naive and memory CD4 cells.

Our research protocol was mainly designed to explore the proliferative responses of memory ($CD4^+/CD29^+$) T lymphocytes in SPCs when they were challenged with two common environmental stimulants (*C. albicans* and SK) and TT, an antigen to which adults are rarely exposed by natural contact.

All of our SPCs showed a significant depletion in the number of $CD4^+$ T cells and $CD4^+/CD29^+$ and $CD4^+/CD45RA^+$ subsets, as initially reported by Vuillier et al. (18). Taken as a whole group, the $CD4^+$ cells from our SPCs were low-level responders to the three soluble antigens, reaching significance

TABLE 2. Proliferative responses to soluble antigens by PBMCs and purified $CD3^+/CD4^+$ and $CD4^+/CD29^+$ cells in patients and controls

Cell preparation	Antigen[a]	Proliferative response[b] in the following subjects:			
		HVs (n = 11)	SNHs (n = 10)	SPCs (n = 14)	AIDS patients (n = 6)
PBMCs	SP	980 ± 384	888 ± 166	1,097 ± 245	730 ± 276
	TT	78,512 ± 28,907	60,312 ± 11,254	30,413 ± 12,416	438 ± 96[c]
	CA	35,951 ± 11,308	19,759 ± 7,115	15,308 ± 5,620	765 ± 348[c]
	SK	15,751 ± 6,017	13,734 ± 7,345	7,062 ± 2,555	1,073 ± 771[c]
$CD3^+/CD29^+$	SP	1,181 ± 711	1,262 ± 291	576 ± 136	682 ± 276
	TT	89,393 ± 33,915	104,718 ± 28,030	42,290 ± 19,523	959 ± 400[c]
	CA	42,802 ± 15,710	20,489 ± 5,270	10,586 ± 3,219[c]	824 ± 281[c]
	SK	12,882 ± 2,153	20,339 ± 12,534	8,287 ± 5,412	606 ± 256
$CD4^+/CD29^+$	SP	1,097 ± 671	1,769 ± 555	749 ± 257	454 ± 241
	TT	65,138 ± 32,033	123,726 ± 34,219	42,301 ± 23,846	421 ± 172[c]
	CA	24,107 ± 8,258	13,224 ± 2,515	9,065 ± 4,667[c]	964 ± 609[c]
	SK	10,367 ± 4,357	16,562 ± 9,316	3,048 ± 1,626[c]	348 ± 113[c]

[a] SP, spontaneous proliferation; CA, *C. albicans*.
[b] Expressed as the arithmetic mean ± standard deviation of counts per minute.
[c] $P < 0.005$.

only in the case of *C. albicans*. However, highly enriched $CD4^+/CD29^+$ memory cells from these patients showed a significantly decreased proliferation in response to both *C. albicans* and SK. Initially, Lane et al. (13) demonstrated that in patients with AIDS, $CD4^+$ lymphocytes failed to respond to TT but were able to express mitogen- and/or alloantigen-driven blast transformation. Subsequently, Giorgi et al. (10) showed that $CD4^+$ subsets were not selectively depleted at any stage of HIV infection and suggested that the loss of the responses to soluble antigens was not related to a particular CD4-cell subset depletion. However, Clerici et al. (6) and Shearer et al. (17) showed among asymptomatic HIV-seropositive patients four distinct patterns of proliferative responses when PBMCs were tested with recall antigens, alloantigens, and mitogens; none of these patterns was related to a critical reduction in the numbers of $CD4^+$ cells.

In our experiments, the qualitative defect (diminished proliferation) in SPCs is most probably related to memory cell dysfunction without the participation of abnormalities in the antigen presentation process, since a similar amount of autologous monocytes was added to each cell preparation. This particular finding was previously suggested by Fauci (9) in studies with accessory cells from identical twins. Furthermore, when each of the SPC individuals was analyzed, we noted that half of the patients responded to one or more of the selected antigens, representing perhaps a very early stage of the HIV infection, while the others did not respond to either antigen. The numbers of $CD4^+$ T cells and $CD4^+/CD29^+$ and $CD4^+/CD45RA^+$ subsets were similar in both R and NR individuals. Our two SPC subgroups (R and NR) may parallel the short-term and long-term HIV-seropositive asymptomatic individuals reported in 1987 by Giorgi et al. (10).

In relation to the susceptibility of $CD4^+$-cell subsets to HIV, Schnittman et al. (16) showed a preferential defect of memory $CD4^+/CD29^+$ T cells in their response to TT, being the abnormality directly related to a greater burden of HIV among these cells. Within this context, Cayota et al. (4) demonstrated that both naive and memory $CD4^+$-cell subsets are impaired in patients with HIV infection.

Therefore, with the data accumulated in several laboratories, including ours, it seems plausible to further advance the hypothesis that HIV indeed interacts not only with $CD4^+$ T

TABLE 3. Proliferative responses to soluble antigens by PBMCs and purified $CD3^+/CD4^+$ and $CD4^+/CD29^+$ cells among SPCs in the R and NR groups

Cell preparation	Antigen[a]	Proliferative response[b] in the following subjects:		
		HVs (n = 11)	SPCs	
			R (n = 7)	NR (n = 7)
PBMCs	SP	980 ± 384	1,654 ± 377	539 ± 117
	TT	78,512 ± 28,907	59,830 ± 19,469	995 ± 673[c,d]
	CA	35,951 ± 11,308	29,734 ± 8,209	881 ± 314[c,d]
	SK	15,751 ± 6,017	12,550 ± 3,636	659 ± 331[c,d]
$CD3^+/CD29^+$	SP	1,181 ± 711	762 ± 253	398 ± 64
	TT	89,393 ± 33,915	82,953 ± 33,166	1,626 ± 653[c,d]
	CA	42,802 ± 15,710	20,566 ± 3,413	606 ± 218[c,d]
	SK	12,882 ± 2,153	14,759 ± 9,657	1,350 ± 574[d]
$CD4^+/CD29^+$	SP	1,097 ± 671	1,144 ± 476	353 ± 188
	TT	65,138 ± 32,033	83,124 ± 43,682	1,479 ± 668[c]
	CA	24,107 ± 8,258	17,548 ± 8,388	581 ± 249[c,d]
	SK	10,367 ± 4,357	5,165 ± 208	578 ± 252[d]

[a] SP, spontaneous proliferation; CA, *C. albicans*.
[b] Expressed as the arithmetic mean ± standard deviation of counts per minute.
[c] $P < 0.05$ (R versus NR).
[d] $P < 0.05$ (R and NR versus HVs).

TABLE 4. Phenotypic analysis of T cells and T-cell subsets in HVs and SPCs in the R and NR groups

Cell	Amt (mm^3 [%]) of fluorescent cells in the following subjects[a]:		
	HVs (n = 20)	SPCs	
		R (n = 7)	NR (n = 7)
Leukocytes	8,358 ± 2,745	9,407 ± 2,850	7.314 ± 3.465
Lymphocytes	3,164 ± 978 (39 ± 10)	3,148 ± 1,115 (35 ± 15)	2,932 ± 1,472 (39 ± 6)
$CD3^+$	2,237 ± 612 (72 ± 7)	2,348 ± 907 (75 ± 17)	2.501 ± 1.550 (81 ± 14)
$CD29^+$	2,349 ± 821 (74 ± 12)	2,150 ± 813 (69 ± 16)	2,402 ± 1,432 (80 ± 14)
$CD45RA^+$	2,243 ± 766 (72 ± 13)	2,176 ± 736 (70 ± 10)	2,273 ± 1,284 (76 ± 11)
$CD3^+/CD29^+$	1,772 ± 646 (53 ± 12)	1,531 ± 871 (51 ± 26)	1,975 ± 1,453 (61 ± 22)
$CD3^+/CD45RA^+$	1,538 ± 625 (50 ± 15)	1,487 ± 619 (48 ± 19)	1,876 ± 1,334 (59 ± 18)
$CD4^+$	1,335 ± 500 (43 ± 8)	888 ± 402[b] (28 ± 6[b])	772 ± 428[b] (22 ± 7[b])
$CD4^+/CD29^+$	998 ± 377 (31 ± 9)	472 ± 246[b] (15 ± 6[b])	502 ± 355[b] (17 ± 6[b])
$CD4^+/CD45RA^+$	759 ± 426 (25 ± 11)	485 ± 225[b] (16 ± 7[b])	499 ± 489[b] (15 ± 6[b])
$CD8^+$	899 ± 326 (29 ± 8)	1,551 ± 745[b] (49 ± 16[b])	1.821 ± 1.232[b] (57 ± 15[b])
$CD4^+/CD8^+$	1.62 ± 0.6	0.6 ± 0.3[b]	0.4 ± 0.2[b]

[a] Results are expressed as the arithmetic mean ± standard deviation of absolute amount (cubic millimeters) and percentages of fluorescent cells by flow cytometry.
[b] $P < 0.005$ when compared with HVs.

cells but also with $CD4^+$ T cells in both evolving stages of maturation, the naive and memory $CD4^+$ T-cell subsets. Two main and probably independent immunopathological lesions arise: the installation of an impaired lymphocyte proliferative response capability and a progressive depletion of the $CD4^+$ pool and its subsets. As we have shown, the decreased proliferation in response to common environmental antigens (i.e., *C. albicans* and SK) seems to be early evidence of the proliferative abnormality; this is followed in asymptomatic individuals by the loss of a response to less common soluble antigens (i.e., TT). Moreover, there seems to be no correlation in the asymptomatic stage of the infection between the diminution of the proliferative responses and the degree of depletion of $CD4^+$ lymphocytes. Both immune abnormalities probably would act as inducers of the state of secondary systemic anergy (1) which is characteristic of the clinical phases of HIV infection. In conclusion, the assessment of T-cell proliferation in response to common environmental antigens might prove useful when evaluating the impairment of immune response in HIV-infected individuals.

ACKNOWLEDGMENTS

This work was funded by the Consejo de Desarrollo Científico y Humanístico, Universidad Central de Venezuela (CDCH-10.31.2294.90) and by Programa Genic (CONICIT-CNRIC).

REFERENCES

1. **Bianco, N. E.** 1992. The immunopathology of systemic anergy in infectious diseases: a reapraissal and new perspectives. Clin. Immunol. Immunopathol. **62:**253–257.
2. **Bowen, D., H. Lane, and A. Fauci.** 1985. Immunopathogenesis of the acquired immunodeficiency syndrome. Ann. Intern. Med. **103:**704–709.
3. **Boyum, A.** 1968. Separation of leucocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97)**:**77–89.
4. **Cayota, A., F. Vuillier, D. Scott-Algara, V. Fenillie, and G. Dighiero.** 1993. Differential requirements for HIV-1 replication in naive and memory CD4 T cells from asymptomatic HIV-1 seropositive carriers and AIDS patients. Clin. Exp. Immunol. **91:**241–248.
5. **Centers for Disease Control.** 1986. Classification system for human T-lymphotropic virus type III/lymphadenopathy associated virus infection. Morbid. Mortal. Weekly Rep. **35:**334–340.
6. **Clerici, M., A. Landay, H. Kessler, R. Zajac, R. Boswell, S. Muluk, and G. Shearer.** 1991. Multiple patterns of alloantigen presenting/stimulating cell dysfunction in patients with AIDS. J. Immunol. **146:**2207–2213.
7. **De Martini, R., R. Turner, and S. Formenti.** 1988. Peripheral blood mononuclear cell abnormalities and their relationship to clinical course in homosexual men with HIV infection. Clin. Immunol. Immunopathol. **46:**258–271.
8. **Echeverria, G., L. Deibis, C. Garcia, T. Olaria, M. Márquez, I. Blanca, and N. E. Bianco.** 1989. Immunopathogenic aspects of infection by the human immunodeficiency virus in Venezuela. PAHO Bull. **23**(1–2)**:**68–75.
9. **Fauci, A.** 1987. AIDS: immunopathogenic mechanisms and research strategies. Clin. Res. **35:**503–510.
10. **Giorgi, J., J. Fahey, D. Smith, L. Hultin, H. Cheng, R. Mitsuyasu, and R. Detels.** 1987. Early effects of HIV on CD4 lymphocytes in vivo. J. Immunol. **138:**3725–3730.
11. **Gruters, R., F. Terpstra, R. De Jong, C. Van Noesel, R. Van Lier, and F. Miedema.** 1990. Selective loss of T cell functions in different stages of HIV infection. Eur. J. Immunol. **20:**1039–1044.
12. **Julius, M., E. Simpson, and L. Herzen Berg.** 1973. A rapid method for isolation of functional thymus derived murine lymphocytes. Eur. J. Immunol. **3:**645.
13. **Lane, C., J. Depper, W. Greene, G. Whalen, T. Waldmann, and A. Fauci.** 1985. Qualitative analysis of immune function in patients with the acquired immunodeficiency virus. N. Engl. J. Med. **313:**79–84.
14. **Morimoto, C., N. Letvin, A. Boyd, M. Hagan, H. Brown, M. Kornacki, and S. Schlossman.** 1985. The isolation and characterization of the human helper inducer T cell subset. J. Immunol. **134:**3762–3769.
15. **Morimoto, C., N. Letvin, J. Distaso, W. Aldrich, and S. Schlossman.** 1985. The isolation and characterization of the human suppressor inducer T cell subset. J. Immunol. **134:**1508–1515.
16. **Schnittman, S., C. Lane, J. Greenhouse, J. Justement, M. Basele, and A. Fauci.** 1990. Preferential infection of $CD4^+$ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T cell functional defects observed in infected individuals. Proc. Natl. Acad. Sci. USA **87:**6058–6062.
17. **Shearer, G., D. Bernstein, K. Tung, C. Via, R. Redfield, S. Salahuddin, and R. Gallo.** 1986. A model for the selective loss of major histocompatibility complex self restricted T cell immune responses during the development of acquired immune deficiency syndrome (AIDS). J. Immunol. **137:**2514–2521.
18. **Vuillier, F., C. Lapresle, and G. Dighiero.** 1986. Comparative analysis of CD4-4B4 and CD4-2H4 lymphocyte subpopulations in HIV negative homosexual, HIV seropositive and healthy subjects. Clin. Exp. Immunol. **71:**8–12.
19. **Wysocki, L., and V. Sato.** 1978. "Panning" for lymphocytes: a method for cell separation. Proc. Natl. Acad. Sci. USA **75:**2848.