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# Histochemistry of Receptors

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# 5 Receptors in Lymphoid Cells

## 5.1 Cell surface receptors in lymphoid cells: from cytochemistry to molecular biology and from a phenotype to a function

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Conclusive evidence for the definite role of lymphocytes in immune responses has existed since the late 1950s. Lymphocytes are the cellular elements responsible for the specific reactions against antigens. These responses can be divided into «humoral» and «cell-mediated» immunity.

Humoral immunity means that the effector mechanism leading to the elimination of antigens is mediated by molecules in such organic liquids as blood, saliva, milk, etc.; these molecules are immunoglobulins or antibodies. They are secreted by plasma cells; these cells derive from lymphocytes, which proliferate and undergo differentiation after antigenic stimulation. The cells responsible for humoral immunity have been defined as «B» lymphocytes; «B» stands for «Bursa» derived, as defined from studies in chickens; in fact in adult mammals B lymphocytes are formed in bone marrow. The antigens which are the targets of antibodies are either free proteins, glycoproteins or peptides, or are inserted in cell membranes. The fixation of antibodies to antigens result in precipitation, neutralization, complement activation, etc., leading to the elimination of the antigen.

The effectors of «cell-mediated» immune responses are lymphocytes. This subset of lymphoid cells acquires the capacity to destroy the target antigens. These antigens are located on cell membranes. The recognition of the antigens by the lymphocytes results in cell lysis («cytotoxicity»). These effector lymphocytes are found distributed among the T cell population. «T» stands for thymus; indeed T cells originate in bone marrow precursors, which home into the thymus where they proliferate and differentiate into T cells which then migrate to the peripheral lymphoid tissues.

Besides the effector cytotoxic T cells (Tc) there are also T cell subsets with regulatory functions: the T helper cells (Th) are involved in the activation of B and T cells after antigen recognition; whereas T suppressor cells (Ts) are responsible for negative signals that inhibit the immune responses.

The complexity of the immune responses led investigators to concentrate on identifying the lymphoid cell subsets responsible for the different functions mentioned above.

The first approach was purely morphological, it was based on the topography of the lymphoid populations in the lymphoid organs as well as on cytology by light and electron microscopy. For example, scanning electron microscopy permitted definition of several cell surface differences between B- and T-lymphocytes, such as the presence of tiny microvilli(POLLIACK et al. 1973, 1976). However, these morphological criteria were not easy to use and were not perfectly reproducible.

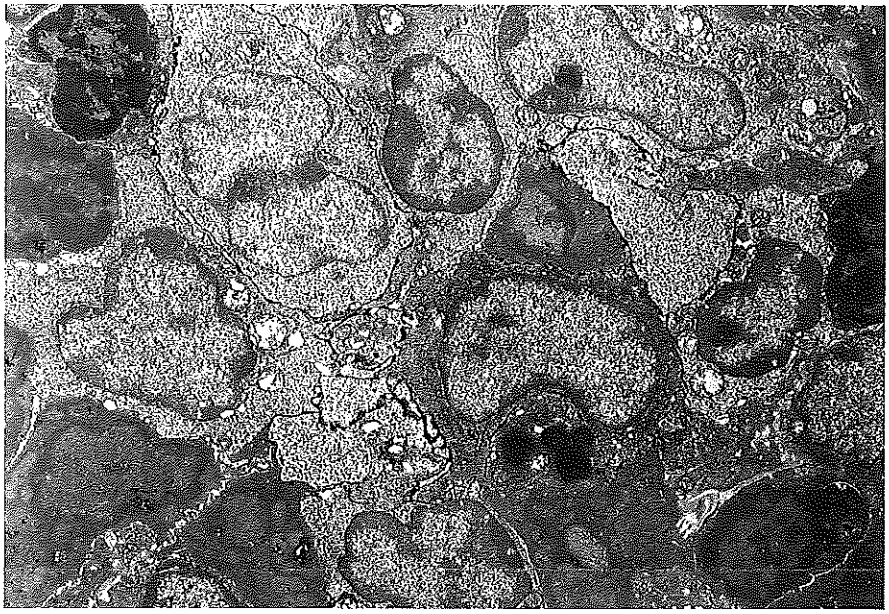

Fig. 1. Alkaline phosphatase in the thymus cortex of C57BL/Ka mice. Ultrastructural cytochemistry was performed as described (GOFFINET et al. 1983). A few large lymphocytes are positive.

A second approach consisted of identifying cell surface molecules, which might be markers of the various lymphocyte subsets. Lymphoid plasma membranes sustain some *enzymatic* activities. To give an example, alkaline phosphatase activity was reported by several authors in thymic lymphomas that spontaneously developed in AKR mice or else were experimentally induced in C57BL mice (SMITH 1961; LAGERLOF and KAPLAN 1967) (Figs. 1 and 2). In fact, in the normal T cell lineage, most foetal thymocytes also express APasé activity; whereas in the adult, only a subset of thymocytes and T cells are positive. In the B cell lineage, membrane APase is also found in some lymphomas and in most activated B cells of normal adults. The enzyme can be detected by cytochemical methods and visualized by microscopy or flow cytometry (LAGERLOF and KAPLAN 1967; DOLBEARE 1980; GOFFINET et al. 1983) or by monoclonal antibodies (MARQUEZ et al. 1990). Little is known of the role of plasma membrane APase in lymphoid cells.

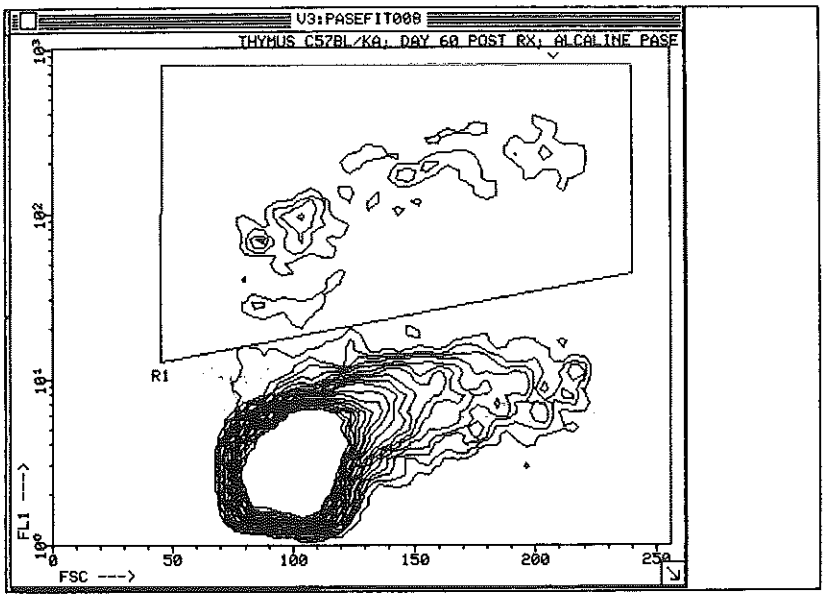


Fig. 2. Alkaline phosphatase in thymocytes 60 days after inoculation of Radiation Leukemia Virus in C57BL/Ka mice. The enzyme was revealed by the cytochemical method described by DOLBEARE et al. (1980) and adapted by us (BONIVER et al. 1982). Flow cytometric bivariate plot showing fluorescence intensity of alkaline phosphatase activity (FL1) and forward light scatter (FSC), related to cell size. About 4% of thymocytes bear this marker of lymphoma cells (Region R1, see text).

Cell coat components were also studied. One can, recall here the use of polycationic dyes in this connection. As an example, we can mention the use of lanthanum ions to show the presence of *glycoproteins* at the ultrastructural level in resting and in activated T or B-lymphocytes (ANTEUNIS and VIAL 1975). All resting lymphocytes were stained by lanthanum in a rather similar way; however, after mitogenic stimulation, there was a slight decrease in the surface labeling of the T cells; conversely, in the B cells very considerable staining alterations were observed. These observations were of great interest not only because they held out prospects of achieving a differential characterization of B cells and T cells, but also because there was clearly some link between cell surface staining properties and functional capacities.

Similar studies were performed with other cell coat markers such as ruthenium red, periodic acid silver methenamine, colloidal thorium, phosphotungstic acid, cetylpyridinium chloride (Fig. 3) and cationized ferritin. This latter method permitted the surface of fixed cells to be stained on the basis of the number and arrangement of anionic groups (such as neuramic acid) on the cell surface (ZEILLER et al. 1976).

The observation that several *lectins* were capable of binding polysaccharidic molecules on the lymphocyte membranes gave rise to another field of research. Thus, B and T cells could be separated by using a panel of lectins with the capacity to bind various kinds of oligosaccharides on the cell membrane (SHARON 1983). As an example, phytohemagglutinin A (PHA) which combines with acetylgalactosamine was found to bind T cell membrane but not B cells (SHARON

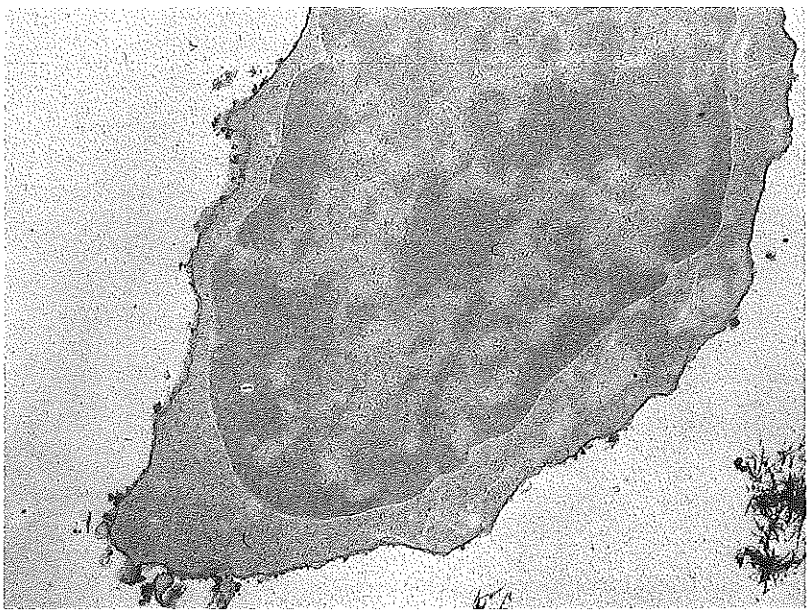

Fig. 3. Cross sectional electron micrograph of a thymocyte labeled with cetylpyridinium chloride (CPC) and ferric thiocyanate. By this method, cell-coat polyanions are bound with quaternary ammonium salt (CPC); the resulting complex is revealed with ferric thiocyanate. The reactions are performed in the presence of electrolytes; the CPC-glycocalix complex is solved if salt concentration is equal or superior to 0.1 M, indicating the presence of carboxyl groups of sialic residues in the cell coat (COURTOY et al. 1974).

1982). Fluorochromes, ferritin and horseradish peroxydase were used to reveal lectin binding and thus to visualize the labeled cells (Fig. 4) (NICOLSON and SINGER 1974).

Another major step came with the development of *antisera* capable of reacting with cell surface molecules which differed in the T and B cells. By using antisera against immunoglobulins, B cells were shown to bear immunoglobulin molecules on the plasma membrane. Besides, the presence of theta antigen (Thy 1) was also detected on cells of the T lineage in mice (REIF and ALLEN 1966). These antisera were then widely used in many procedures. In combination with electron microscopy, they permitted a better morphological characterization of B and T cells (AOKI et al. 1960; DE PETRIS and RAFF 1972; SANTER et al. 1972). They were also very helpful in defining more accurately the functional properties of the various lymphocyte populations.

The most recent breakthrough came from the *hybridoma* technology developed by KOHLER and MILSTEIN (1975). Several workers applied this methodology very early on in order to obtain *monoclonal antibodies* reacting with lymphoid cells.

The most fruitful approach in this early time gave rise to the development of antilymphocyte monoclonal antibodies. Interestingly, these antibodies were first related to the T cell populations (KUNG et al. 1979; KUNG and GOLDSTEIN 1980); some of them obviously react with most if not all mature T cells whereas others react only with some mature T cells. As an example, the OKT3 or anti-Leu 4 monoclonal antibody recognizes most of mature T cells. At the opposite end of the

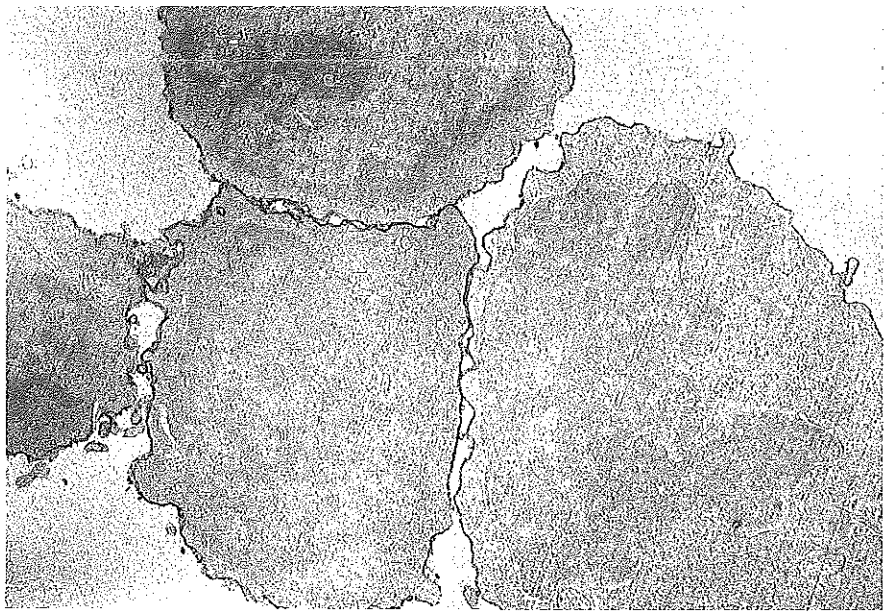

Fig. 4. Cross sectional electron micrograph of murine thymic lymphoma cells labeled with concanavalin A-horseradish peroxydase diaminobenzidine, as described (BARAT and ARAMEAS 1972).

scale, the OKT4 (or anti-Leu 3) monoclonal antibody reacts with 60–70% of peripheral blood T cells and the OKT8 (or anti-Leu 2) with only 30 to 40% of blood T lymphocytes; in fact, the expression of the antigens recognized by these antibodies is mutually exclusive in peripheral T cells (Fig. 5).

The OKT4 and OKT8 monoclonal antibodies allowed the segregation of two subsets of T lymphocytes which indeed displayed different functional capacities: OKT4 bound helper T cells, whereas OKT8 labeled cytotoxic or suppressor T cells (RHEINHERZ et al. 1979; LEDBETTER et al. 1981). This led to a very active use of these monoclonal antibodies and of others produced later on. The definition of the ratio between OKT4+ cells/OKT8+ cells in peripheral blood was shown to be a reflection of the immune status: hence more and more clinical indications appeared, such as the follow up of organ transplantation or of HIV-infected patients. Let us recall here that the observation of a low OKT4/OKT8 ratio is an early immunological marker of a HIV induced acquired immunodeficiency syndrome, manifested before the onset of any opportunistic infection or associated cancer (STAHL et al. 1982).

The amount of monoclonal antibodies reacting with blood leukocytes became so high that a need for an international consensus for defining the recognized cell surface antigens became apparent. Most of people working in the field met and decided to designate as «cluster differentiation antigens» the cell surface molecules recognized by the monoclonal antibodies. As an example, the molecular structure recognized by OKT4 or anti-Leu 3 was termed CD4 while that recognized by OKT8 or anti-Leu 3 was named CD8.

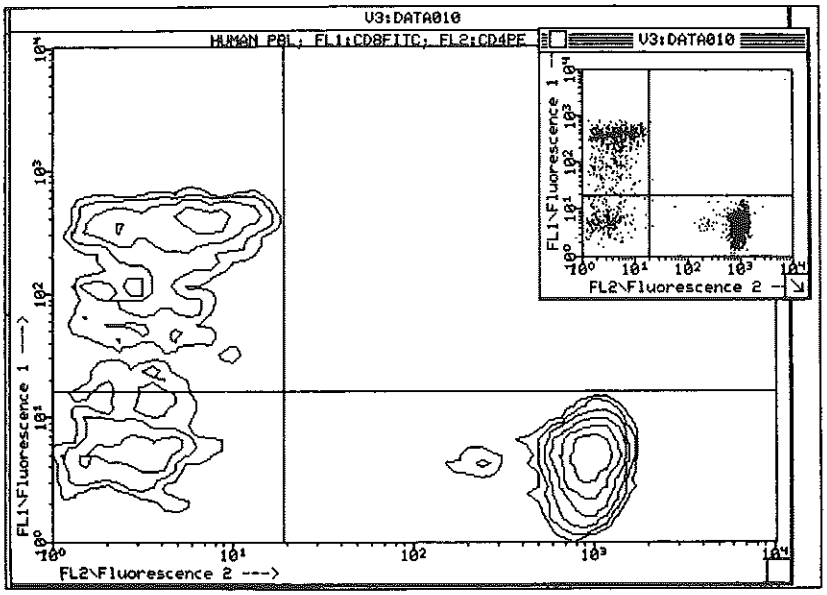


Fig. 5. Distribution of CD4+ and CD8+ T-lymphocytes in human peripheral mononuclear cells (PBL). Cells were stained with FITC conjugated anti CD8 monoclonal antibodies and phycoerythrin conjugated anti-CD4 monoclonal antibodies. Flow cytometry bivariate plot of CD4+ (Fl2) and CD8+ (Fl1) cells. T cells in PBL are either CD4+ or CD8+.

Simultaneously to the production of these antibodies by the hybridoma technology, more and more methods were established to identify the cell surface structures recognized by the monoclonal antibodies.

A first approach was mostly used by the morphologist: the sample was either a cell suspension, or a cell smear or cytospin, or cultured adherent cells, or frozen or paraffin tissue sections. Antibody binding was visualized with reagents which were labeled either with fluorochromes, or enzymes, or radioactive iodide, or colloidal gold. These reagents were either directly or indirectly conjugated to the monoclonal antibodies. The indirect procedure may be very complex and involves either second, third or fourth party antibodies or else the biotin-avidin complex (Review: Harlow and Lanf 1988).

The observation procedure is the light microscope (fluorescence microscopy or routine light microscopy, possibly used with special devices such as epipolarization or darkfield for the immunogold-silver staining: De Waele et al. 1986) or transmission or scanning electron microscope (Fig. 6).

A second approach was extensively developed by the cell immunologists who needed a fast procedure to obtain quantitative data from their monoclonal antibody-stained samples. Flow cytometry, which was developed in the 70 s in the U.S.A., rapidly became the tool of choice for that purpose (Review: Parks et al. 1989). It allows stained cell suspensions to be analyzed at a very high rate (about 10,000 cells are currently analyzed in 20 sec). It requires the use of fluorochrome labeled reagents (Fig. 5). Thanks to constantly upgraded equipment and the availability of

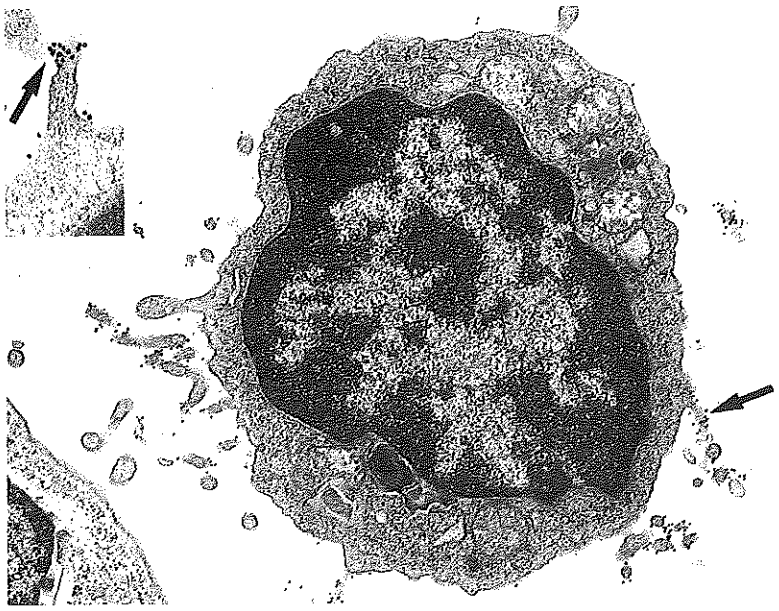

Fig. 6. Immunogold identification of Thy 1.2 antigen. Cross sectional electron micrograph of a murine thymocyte labeled with anti-Thy 1.2 monoclonal antibody (mouse IgM) and 20 nm gold particle conjugated goat anti mouse IgM antiserum.

various kinds and combinations of fluorochromes, two or three different antigens can now be detected simultaneously on a single cell. The major criticism against this method is that the cells are not morphologically visualized by the observer. However, this can be partially circumvented if one uses the sorting capacity of the flow cytometer: monoclonal antibodies labeled cell subsets can be sorted on the basis of a cell surface phenotype and then analyzed by morphological means.

It is thus clear that the visualization of membrane molecules in lymphocytes is a very efficient way to segregate the various subsets of lymphoid cells by their «surface phenotype».

Interestingly, in connection with this practice of phenotyping lymphoid cells, it appeared very clearly that the membrane molecules recognized by the various ligands we have described are involved in the functional activity of the lymphocytes; indeed, most of these molecules act as receptors for physiological ligands and binding the ligands to the receptor lead to lymphocyte activation, including proliferation and differentiation into immunocompetent functional cells.

A prime example is that of lectins; as mentioned above, lectins can react specifically with carbohydrate residues of polysaccharide chains on the plasma membranes of lymphocytes and thus are useful tools for distinguishing some subpopulations of lymphocytes. Besides, lectin binding to living lymphocytes can result in mitogenic stimulation. A good example is that of phytohaemagglutinin, which reacts with N-acetylgalactosamine residues. PHA induces the proliferation of T cells; in other words, PHA binding on the T cell membrane emits a mitogenic signal (Fig. 7). The activated cell divides and can express various functional activities (SHARON 1983).

Even more interesting is the role of cell surface molecules in antigen binding, which is a critical and essential step in the immune response. In B cells surface immunoglobulins act as antigen receptors. These immunoglobulins are composed of two light and two heavy chains encoded by several genes located on several chromosomes. Interestingly, each B cell bears a unique type of immunoglobulin characterized by the molecular structure of its terminal part, named «variable region». The variability of the immunoglobulin molecule is not genetically transmitted: rather it is acquired after birth by gene rearrangement and mutation occurring in maturing B cells. It takes into account the remarkable potentially infinite repertoire of antibodies specificities (Review: Calame 1985; Yancopoulos 1986).

When a B lymphocyte binds its specific antigen through a cell surface immunoglobulin, it undertakes the first step of immune function: recognition. This recognition step leads to B cell activation through the phosphatidyl-inositol and protein kinase C pathways (Cambier and Ranson 1987). Furthermore, antigen loaded B cells can act as antigen presenting cells for helper T cells (Mohler 1975).

T lymphocytes also bear cell surface molecules which can act as antigen receptors: these molecules are disulfur-bound heterodimers which are composed of a constant and a variable region (Review: Kronenberg et al. 1986). Most of mature T cells bear a receptor (Ti) composed of $\alpha$- and $\beta$-chains, but some mature T cells bear a $\gamma/\delta$ receptor. The variable regions of these molecules are also somatically acquired and result from gene rearrangements. They can be demonstrated in lymphocyte by using molecular biology to show DNA rearrangements. Here again, ligand (in this case, antigens) binding to the receptors leads to cell activation (Review: Weiss and Imboden 1987).

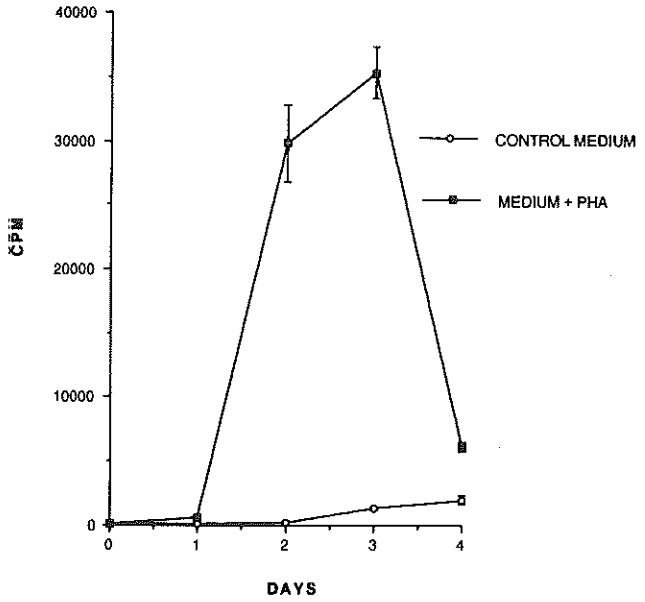


Fig. 7. PHA-induced lymphocyte activation. Human PBL were cultivated in the presence of PHA (1 mg/ml) and cell proliferation was determined by cpm counts after $^{3}$H-thymidine incorporation.

One of the antigen classically identified on most of mature T cell is CD3; monoclonal antibodies such as OKT3, anti-Leu 4, BMA 030, ..., are used for that purpose. Beside this interest in counting the total number of mature T cells or in isolating T cells from a biological sample, anti-CD3 binding to CD3 on the plasma membrane of T cell results in several interesting functional effects. Initially, the antibody was used in order to obtain lymphoid cell suspensions depleted from T cells; indeed, incubation of T cells with anti-CD3 and complement results in the lysis of the CD3+ cells. Then several groups of clinicians and researchers thought that anti-CD3 monoclonal antibody could be used in vivo in order to control T cell mediated immunopathological reactions such as graft rejection (Cosimi et al. 1981a, 1981b). It was demonstrated that OKT3 can control or even prevent, acute graft rejection in kidney, liver or heart transplantation. However, several intriguing observations were made. If the OKT3 mediated T cell depletion was obvious in the early period after injection, CD3− CD4+ or CD3− CD8+ T cells were later on detected in the blood. In fact, the CD4+ or CD8+ cells were mature but not functional; the lack of CD3 on the plasma membrane was not due to a defect of differentiation but to the elimination of CD3 cell surface molecules after binding with the monoclonal antibody («antigenic modulation») (Chatenoud et al. 1982). This observation suggested that CD3 on the plasma membrane was involved in T cell functions. Further studies clearly showed that small amounts of anti CD3 monoclonal antibody added to T cells induced the activation of these T cells, undergoing cell division (Fig. 8) and even acquiring some such functions as lymphokine secretion or cytotoxic activities (Fig. 9) (Van Wauwe and Goossens 1981; Oettgen and Terhorst 1987; Anderson et al. 1988; Chatenoud et al. 1990; Moutschen et al. 1990). Subsequent studies demonstrated that anti-CD3 binding to CD3 resulted in activation of the phosphatidylinositol pathway. Other investigations were devoted to the identification of the physiological ligand for membrane CD3. It was shown that CD3 is indeed closely associated to the T cell receptor heterodimer (α/β or γ/δ)

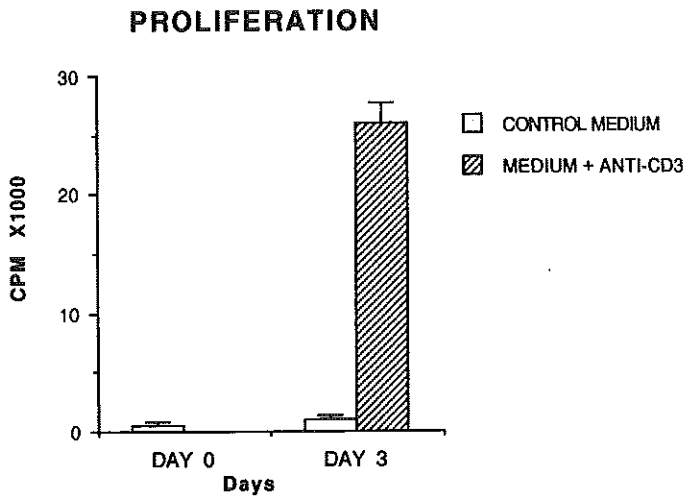


Fig. 8. Anti-CD3 induced proliferation: $^{3}$H-thymidine incorporation by fresh peripheral blood lymphocytes (PBL) and PBL cultured in control medium or with anti-CD3 mAB (BMAO30 100 ng/ml). Means and standard deviation are given for 3 separate experiments.

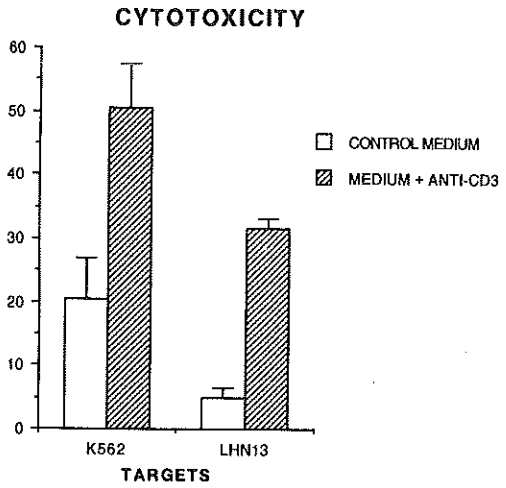


Fig. 9. Anti-CD3 induced cytotoxicity: anti-K562 and anti-LHN13 cytotoxic activities of PBL on day 3 in a 4 h Chromium release assay. The effector cells were cultured in control medium or with anti-CD3 mAB (BMAO30 100 ng/ml). K562 and LHN13 are cancer cell lines frequently used as targets for cytotoxic lymphocytes in the *in vitro* assays.

on the cell membrane (Samelson et al. 1985). In fact, when antigen binds to the receptor, the activation signal is transduced to the cell through the CD3 molecule (Oettgen and Terhorst 1987).

The complexity of the interaction between T cells and antigenic structures is still more complicated and again related to the cell surface receptors. We mentioned earlier that mature T cells express either the CD4 molecule in helper T cells or the CD8 molecule in cytotoxic-suppressor T cells. Furthermore, the interactions of lymphocytes with antigen is «MHC restricted» (MHC = major histocompatibility complex). MHC gene products are involved in the cooperation between T and B cells, which are required to trigger antibody production after antigen recognition. Indeed, the helper T cell has to recognize self class II MHC molecules on the surface of B cells; if this requirement is fulfilled, the B cell can be stimulated to proliferate and to transform into antibody producing cell (Kindred and Shreffler 1972; Katz and Hamaoka 1973).

Besides, recognition of the antigen by helper T cells and cytotoxic T cells is also MHC restricted (Rosenthal and Shevach 1973; Zinkernagel and Doherty 1974, 1979). T helper cells can recognize antigens on the antigen presenting cell (APC; macrophage) only if this latter cell expresses class II molecules of the same MHC as the T cells, whereas cytotoxic T cells do so on the APC or on the antigenic target cell only if these cells express class I MHC molecules. The antigen is presented to the T cell in a sort of «groove» made by the extracellular part of the MHC molecule (Bjorkman et al. 1987a, b).

In T helper cells, CD4 is closely associated to the (Ti-CD3) TCR complex, whereas in cytotoxic T cells CD8 is closely associated to the (Ti-CD3) TCR complex. CD4 interacts with the class II MHC molecule on the APC, and CD8 with the class I MHC molecule on the APC or on the antigenic target cell. In other words, T helper cell activation requires interaction of a complex made of CD4 and (Ti-CD3) with a comples made of antigen and class II MHC molecule whereas

cytotoxic T cell activation requires interaction of the complex composed of CD8 and (Ti-CD3) with a complex formed class I MHC molecule complex and the antigen (Review: UNANUE 1989).

It should be mentioned that cell surface CD3 is also involved in the T cell differentiation process, which physiologically occurs in the thymus (DEFRESNE et al., in this volume). Thus, the CD3, CD4 and CD8 molecules, which are very useful differentiation markers for T cell subsets, are indeed essential to their functions – at the level of ontogeny, antigen recognition, self to non self discrimination, and cellular activation.

There are many other cell surface molecules on lymphocytes than those cited in this paper. We should mention the receptors for lymphokines, which are expressed at the earliest phase of the activation process, the adhesion molecules and the molecules which are required for lymphocyte traffic. The chosen examples clearly demonstrate the evolution of the studies of lymphocyte plasma membrane: in the early period and indeed still at the present time, the cell surface molecules have been useful for characterizing the different subsets of lymphocytes, i.e. as differentiation markers. The observation that many of these molecules are in fact receptors for physiological ligands makes them particularly interesting for the study of lymphoid functions, i.e. as markers of function and activation. If the approach was initially morphological, it has now reached the molecular level.

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