Analysis of Lymphocyte Proliferation by Cytofluorometric Techniques in Aging and Various Clinical Conditions*

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Lymphocyte proliferation and differentiation represent basic functions of the immune system, since they are required for the acquisition of immunological memory as well as for clonal expansion of specific lymphocyte populations and the production of specific antibodies. In recent years, the combination of several techniques, such as the separation of various lymphocyte and lymphoid cell populations on the basis of membrane markers, the analysis of the cell cycle by cytofluorometry,[1,2] the measurement of ^{3}H-thymidine uptake, the detection of membrane receptors and markers by immunofluorescence or ligand binding[3] and the quantitative assessment of various lymphokines produced by proliferating lymphoid cells have permitted the establishment of an integrated picture of the various events associated with the proliferation and differentiation of lymphocytes. Although this picture is still fragmentary, the analysis of lymphocyte functions in terms of an integrated interplay between lymphocyte-derived factors and lymphocyte receptors (so-called lymphokine "cascades") has already been found relevant and informative in several clinical situations, in which a dysregulation of lymphocyte functions is apparent.

The purpose of this paper is to review briefly the current, more sophisticated possibilities for analysing lymphocyte proliferation and differentiation in clinical situations.

1. Dynamic analysis of lymphocyte proliferation: an integrated approach

Techniques enabling the staining of lymphoid cells simultaneously for intracellular DNA and RNA make it possible to quantify and follow lymphoid cells undergoing proliferation and to assess them throughout various phases of the cell cycle by cytofluorometry.[1,2] A dynamic follow up during the first 48 hours of culture, i.e. while most of the cells have only completed their first proliferative cycle, reveals that several signals and concomitant events are required for the cell to proceed from a resting Go phase through an early activation phase (G1a), a late activation phase (G1b), a DNA synthesis phase (S), a premitotic phase (G2) and mitosis (M).[4]

It is by now well recognized that most substances formerly designated as T-cell "mitogens", such as lectins (e.g. PHA, Con A), are indeed only providing a first activating signal enabling the cell to initiate RNA synthesis and to proceed from the resting Go phase to an early G1a phase.[5,6] In order to proceed further, the cell must receive a second signal provided by interleukin 2 (IL 2), also known as T-cell growth factor (TCGF).[7] The main but possibly not the only effect of this lymphokine seems to be to promote further RNA synthesis, which is manifested by the passage of the cells from the G1a to the G1b phase.[8-10] The postulate that the G1 phase can be subdivided not only quantitatively but possibly also qualitatively into separate subphases at first encountered some skepticism among some molecular biologists. However, some manipulations in cell culture, such as the presence of dexamethasone, which prevents IL1[11,12] and IL 2 production[13] and thereby IL 2/IL 2 receptor interaction, indeed stops RNA synthesis in the cells; the addition of the IL 2 signal triggers anew RNA synthesis, as can be assessed by cytofluorometry[14,15] as well as by biochemical means. (Bettens *et al*, manuscript in preparation).

While some T-lymphocyte populations seem to produce IL 2 constantly and may even be activated by IL2 produced endogenously

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without the participation of a membrane receptor available for exogenous IL 2,[16] most activated T lymphocytes appear to be dependent for their continued proliferation upon the interaction of some exogenous IL 2 with a specific IL 2 membrane receptor formed during the early G1a phase.[7,15,16] The formation of an IL 2 receptor during the activation of lymphocytes, which was originally followed indirectly from IL 2 absorption studies[17] and by direct binding studies with labelled IL 2[18] has recently been kinetically followed in our laboratory and those of others by cytofluorographic studies[15,16,19] with a monoclonal antibody (anti-Tac) recognising a membrane protein identical or closely associated with the IL 2 receptor.[20-24] The appearance of IL 2 receptors follows closely the increase in RNA synthesis characterising the G1a phase; the receptors become detectable by immunofluorescent anti-Tac binding[15] or by direct labelled IL 2 binding[19] approximately 2-4 hours after stimulation; the number of T lymphocytes carrying IL 2 receptors and the density per cell of such receptors increase progressively for the next 18-24 hours. At all phases, the density of IL 2 receptors appears to remain very heterogeneous in the stimulated cell population. Production of IL 2 is apparently the function of some subsets of activated T cells.[25-27] As judged from kinetic analysis, IL 2 becomes detectable in the supernatant of activated lymphocyte cultures about 10-12 hours after the activating signal produced by a lectin.[13] The production of IL 2 in these activated T lymphocytes seems to be dependent upon a second signal provided by a monokine, interleukin 1 (IL 1), the product of activated monocytes or macrophages.[7,26,28] IL 1 becomes detectable in the supernatant of activated mononuclear cell populations 2-4 hours after activation.[14]

Following successful interaction of exogenous IL 2 with the IL 2 receptor, there is a new burst of RNA synthesis (G1b phase) in the cells and some six hours later DNA synthesis starts. The passage from the G1b to the S phase was first thought not to require additional signals: all analyses performed on mouse, canine and human lymphocytes had indicated a close correlation between the number of cells in the G1b phase and ^{3}H-thymidine incorporation, and this correlation independent was of the cell source and of varying culture conditions, with or without the addition of serum.[10,29]

However, it has recently been shown[30,31] and confirmed by us (Bettens *et al*, 1984, unpublished experiments) that the interaction of transferrin with its activation-induced transferrin receptor is also essential for the initiation of the S phase and DNA synthesis. Since monoclonal antibodies directed against the transferrin receptor block the proliferative burst induced by the IL 2/IL 2 receptor interaction[30,32] and since the appearance of the transferrin receptor on the lymphocyte membrane following stimulation by PHA follows the appearance of the IL 2 receptor after an approximately two-hour time lag, it is currently assumed that the two events are linked, although no formal proof is yet available.

It seems that early events are really the most decisive with regard to the fate of proliferating lymphocytes; once activated and furbished with the appropriate signals, they might proceed on their committed path throughout the cell cycle. It must be recognised, however, that further events and signals possibly required at later stages of lymphocyte proliferation, such as the completion of the S phase, and elapsing of the G2 phase, and initiation of the M phase, have not yet been analysed as thoroughly for human peripheral blood lymphocytes proliferating *in vitro* as for lymphoid T-cell lines.[33] Following mitosis, cells may either return to a resting Go stage or directly enter a new proliferation cycle, provided the required activation- and proliferation-inducing signals (e.g. IL 2) are continuously present. This is apparently the case for T cells steadily proliferating in the presence of exogenously provided IL 2; in the absence of IL 2 the cells return to a Go phase, in which they remain susceptible to renewed activation.[16,19] Stimulated T cells which have been deprived of IL 2 before entering the S phase and have returned to Go show an accelerated expression of IL 2 receptors upon restimulation;[19] it is tempting to consider such cells as T "memory" cells. Our current dynamic conceptions about cell cycle associated events in human T-lymphocyte proliferation are illustrated in Figure 1.

Since lymphoid cells taken from a living animal may be expected to have been, at least in part, activated *in vivo*, a variable proportion of peripheral blood T lymphocytes are in fact already progressing throughout the cell cycle at time 0. This can be assessed by a ^{3}H-thymidine pulse at time 0[6,34,35] as well as by cytofluorographic analysis. At time 0, ^{3}H-thymidine uptake reflects the activity of cells which have reached the G1b/S phases *in vivo* and, therefore, has also been used clinically as some kind of measure of "immunological activation" *in vivo*. However, its interpretation is somewhat clouded by the observation that in several acute clinical conditions where this parameter (^{3}H-thymidine uptake at To) has been followed, continuously proliferating myeloid cells from the bone marrow appear in the peripheral blood and provide high background values of proliferating cells. Some examples of elevated DNA synthesis at time 0 in peripheral blood lymphocytes have been reported in the literature or are currently being assessed: viral infections, rejection of transplanted kidneys and cases of active chronic hepatitis. A high degree of preactivation *in vivo* is also sometimes apparent from the

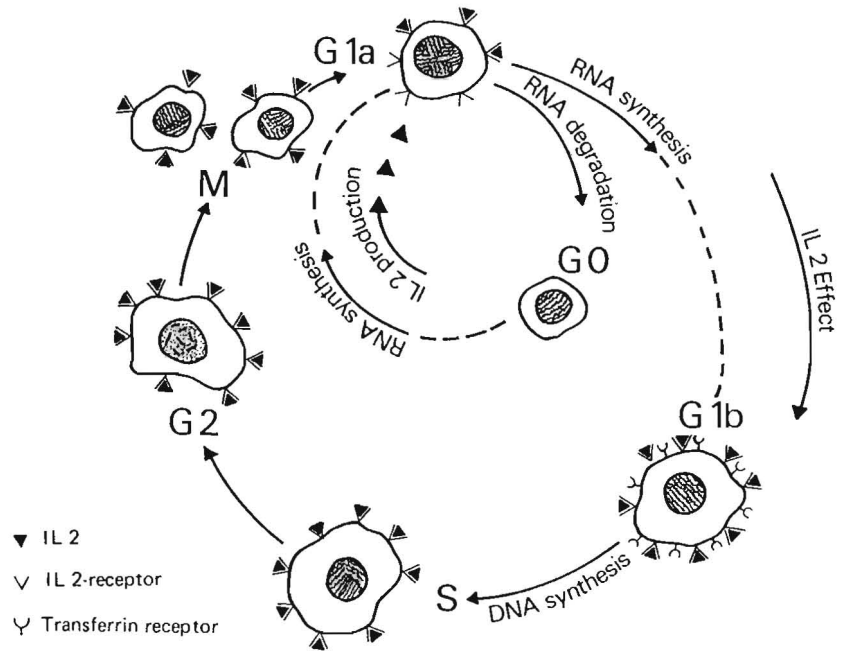

Fig. 1 Lymphocyte cell cycle

high control values observed in non-stimulated lymphocyte cultures pulsed with ^{3}H-thymidine on the third day of culture, provided the endogenous production of IL 2 has been sufficient to sustain proliferation until that time and to permit them eventually to enter a second cell cycle. Cells activated *in vivo* but which have not yet proceeded beyond the G1a phase will proliferate *in vitro* only if they receive the appropriate exogenous IL 2 signal.

It has become evident in recent years that proliferation and differentiation of B lymphocytes requires a cascade of lymphokine/lymphokine-receptors interactions very similar in principle to that described above for T lymphocytes[36] although in part different lymphokines are involved: B-cell growth factor (BCGF) instead of IL 2, T-cell replacing factor (TRF) and possibly several other maturation and differentiation factors, possibly including IL 1 as well.[37] Recently, it has been claimed that IL 2 is required for the production of BCGF,[38] providing thereby one more link between T and B lymphocytes. Up to now, with the exception of assessment of mature B-lymphocyte functions by plaque-forming assays, relatively less attention has been given to the analysis of proliferation and differentiation events in human peripheral blood B lymphocytes. It is obvious, however, that very similar rules will apply in terms of lymphokine and receptor requirements. In particular, it has recently been demonstrated by Fauci's group that large B cells preactivated *in vivo* can also be identified in human peripheral blood and that such cells will proceed without further stimulation to proliferation and differentiation into antibody-secreting cells when provided *in vitro* with the appropriate T-cell factors, such as BCGF, TRF or BMF.[39-41]

The analyses of B-cell activation *ex vivo* in clinical conditions is still in its infancy but might well provide some very useful information. A recent example is the observation that B lymphocytes from patients with acquired immune deficiency syndrome (AIDS) are obviously stimulated *in vivo*, probably by some viruses.[42]

When dealing with the analysis of stimulation *in vitro* by T-cell mitogens, such as PHA or Con A, as has been the case in most of our studies, it is probably safe to assume that the results obtained reflect primarily proliferation of T cells, which also make up the bulk of the peripheral blood lymphocyte population. However, monocytes also activated by lectins and *in vivo* stimulated B cells helped to proliferate by IL 2 and BCGF are potential contributors to the DNA and various RNAs synthesised upon *in vitro* stimulation with T-cell mitogens. Accordingly, it is to be expected that the routine use of more selective stimulating agents, such as anti-T-3 antibody for T cells and anti-μ antibody for B cells, would permit a better assessment of early activating events, the more so as such triggers apparently do not allow the stimulated cells to proceed beyond the first activation events.[39] Activation of lymphocytes to express IL 2 receptors can be achieved in the absence of lectins or antigens by autologous neuraminidase- and glucose-oxidase-treated autologous macrophages.[43]

Since a number of lymphokine-induced intracellular events such as lymphokine production and membrane receptor expression involve RNA and protein synthesis, the question arises at which level the immunoregulatory lymphokines such as IL 1 or IL 2 act intracellularly. There are several examples suggesting that lymphokines may act directly upon the genetic apparatus controlling the synthesis of proteins: e.g. the induction of surface Ig expression and K-light-chain synthesis in pre-B-cell lines by IL 1,[44] the expression of the J-chain gene in activated B cells by T-cell factors,[45] the production of BCGF[36] or gamma interferon[46,47] in T cells by IL 2, the increase in RNA synthesis coding for Ia antigens in macrophages by gamma interferon,[48] the increase in mRNA

for murine γ1 Ig in B cells by the T-cell factor BCDFγ[49] or the expression of IL 2 receptors in T cells by gamma interferon.[50]

2. Analysis of lymphocyte proliferation in aging and various clinical conditions.

As is well known, impairment of various immune functions is a marked feature observed with increasing age in humans as well as in experimental animals.[51-53] It is striking that not all immune functions appear to be affected to the same extent, some even appear to remain essentially intact until a very advanced age. In summary, it might be stated that age affects in particular several T-cell functions and alters the process of self-recognition. Diminution of the proliferation of T cells following lectin stimulation, in particular PHA and Con A, has long since been recognised as a prominent characteristic of immunological aging.[52,54]

Although most studies on the impairment with age of T-cell proliferation have been performed using the ^{3}H-thymidine uptake technique, the integrated approach described above has enabled us to assess more precisely the mechanisms by which T lymphocytes of old mice and elderly humans fail to proliferate as efficiently as the cells of young donors. In order to establish such a lymphocyte proliferation "profile" in a routine clinical test, the following parameters were studied following stimulation by PHA in autologous serum and in a standard AB serum:

- number of G1a and G1b cells (by cytofluorometry)
- incorporation of ^{3}H-thymidine
- effect on ^{3}H-thymidine incorporation of the addition of exogenous IL 2 (10 units/ml) or of indomethacin
- production of IL 2 (in units/ml/24 hrs) under standardised culture conditions.
- number of G1 cells and level of ^{3}H-thymidine incorporation (T 0-4 hours) at time 0.

These investigations permit the assessment of the following: a) the degree of cell activation *in vivo* prior to blood-letting; b) the capacity of the cells to be activated by lectins; c) the production of IL 2; d) the capacity of response to IL 2 presumed to reflect indirectly the state of IL 2 receptors; e) the internal "damping" effect of prostaglandin synthesis presumed to be due essentially to activated monocytes; f) the presence of autologous serum factors affecting the one or the other parameters mentioned above.

Such experiments have been carried out in old inbred and randombred mice[35,55] and in three groups of humans (20-35 years of age, 50-65 years and over 70 years respectively); the results are described in detail elsewhere.[56] In our study and those of others,[57] a decrease in IL2 production and IL 2 receptor expression seemed to be the major event; other authors have pointed to the overall decline in precursor frequency for various T-cell-mediated reactions.[58]

In addition to results obtained with peripheral blood lymphocytes in man, studies on mice have revealed several other age-associated changes, such as modifications in the composition of cellular subsets in various lymphoid organs and differences in lymphocyte compartmentalisation.[59] It is noteworthy that with this more sophisticated approach to the study of aging lymphocytes, age-associated dysfunctions become detectable in middle age. Furthermore, there appears to be increasing individual variations with advancing age. Whether the maintenance of high T-lymphocyte proliferative capacity constitutes a selective advantage for the aging individual has not yet been demonstrated; preliminary prospective studies on a randombred mice population[60] suggest that this may not necessarily be the case. Many other factors, e.g. dysregulation in self-recognition, may influence the overall performance of the immune apparatus in such a way that a slightly lowered T-proliferative capacity would instead represent an adaptive mechanism which could favourably influence survival prospects! On the other hand, marked to complete impairment of T-cell functions, as observed in anergic states and cases of advanced cancer, most probably has a detrimental effect on resistance to infections and thereby on survival. However, in this respect, well-controlled prospective studies are surely needed.

When applied to other clinical conditions or disease groups where immunological dysregulation is postulated to play some role, the analysis of early events in T-lymphocyte proliferation confirms that the various steps in the lymphokine/lymphokine receptor cascade are not obligatorily linked, i.e. profound impairment of IL2 production may be observed in a T-cell population developing a normal IL 2 receptor density profile following mitogen stimulation. In other cases, both IL 2 production and IL 2 receptor density are impaired. In both cases, the net effect is decreased T-lymphocyte proliferation. Such situations have been observed in cases of primary biliary cirrhosis (Reinhardt, Pichler, de Weck and Preising, manuscript in preparation) as well as in cases of other autoimmune disorders. In lepromatous leprosy,[61] the addition of exogenous IL 2 restores the failing production of gamma interferon and the primary defect appears to lie in IL 2 production but not in IL 2 receptor expression. IL 2 abnormalities have been described also in various immunodeficiency conditions[62] and in autoimmune diseases. Since it is postulated by some that the addition of exogenous IL 2 will have some beneficial effects in cases of defective IL 2 production, it is obvious that the ultimate result of such therapy will be profoundly influenced by

the capacity of the treated patient to express IL 2 receptors. In this respect, we should also prepare ourselves for some unexpected surprises. According to experiments performed *in vitro*, the addition of dexamethasone to human lymphocyte cultures profoundly affects IL 2 production but not the rate of IL 2 receptor expression, since dexamethasone-treated cells blocked in the G1a phase of the cell cycle will proceed further to proliferation upon the addition of exogenous IL 2 and they will possess a normal number of IL 2 receptors, as assessed by cytofluorometry with the anti-Tac antibody.[15] However, T lymphocytes from patients treated *in vivo* with prednisone and prednisone derivatives apparently behave in a different way: such lymphocytes are impaired not only in their IL 2 productive capacity but also in their IL 2 receptor expression; such lymphocyes are not reconstituted by the addition of exogenous IL 2.[63]

The rationale for attempting therapy with purified cell line IL 2 or genetically bioengineered IL 2 is the demonstrated possibility of restoring some of the immune defects in cell populations by the addition *in vitro* of IL 2 in cases of AIDS,[64] some immune deficiencies,[65] leprosy[61,66] and cancer.[67] Systemic administration of IL 2 has restored an immune response in T-cell-deficient nude mice,[68] boosted the response to alloantigens in normal[69] and chemotherapeutically depressed mice[70] and inhibited the growth of murine sarcoma cells injected locally together with IL 2.[71] Initial attempts to inject IL 2 systemically in man have shown that this lymphokine causes some transient side-effects such as headache and fever and that it has a fairly short half-life of 5-7 minutes.[72,73] Preliminary clinical evaluation in cases of AIDS and advanced cancer did not indicate clinical improvements or changes in immunological paramaters, such as lymphocyte proliferation, natural killer cell activity or skin reactions to recall antigens.

3. Age- and disease-associated changes in lymphocyte receptors

It is well known among gerontologists that changes in cellular receptors often accompany the aging process and may be directly responsible for altered cellular functions. Age-associated changes in insulin and glucagon receptors in adipocytes,[74] adrenergic receptors in cardiac muscle cells,[75] serotonin receptors in neuronal synapses[76] and various hormonal receptors in different tissues[76] are only some of the examples which may be quoted to illustrate the importance of cellular receptor modifications in aging. It could be stated summarily that "age is a disease of receptors!" Although this is manifestly an overstatement since some lymphocyte receptors do not change with age,[77,78] or may even increase,[76] it may well be that most of the physiological changes accompanying advancing age ultimately rest on modifications in expression, density and/or avidity of cellular receptors.

Studies on aging proliferating lymphocytes have clearly shown that the decreased proliferative capacity rests not only on diminished IL 2 production[27,35,55,79-81] but also on diminished IL 2 receptivity,[55,82] which is itself a consequence of a diminution in the density of IL 2 receptors available on the cell surface. It is not known at this stage whether changes in IL 2 receptor quality (e.g. molecular alterations in receptor structure leading to decreased affinity for IL 2) also participate in the overall decreased efficiency of IL 2 – IL 2 receptor interactions.

These observations have led us[83,84] to investigate the fate of other receptors known to be present on lymphocytes, with regard to the function of the cell cycle on the one hand, and of the age of the cell donor on the other hand. Some of these other receptors seem to appear on the cell membrane only during the G1 phase following activation (e.g. insulin, transferrin),[85-88] others are present on resting Go cells but increase markedly during the G1 phase (e.g. corticosteroid receptors)[89-91] while others apparently change little during the cell cycle (OKT4 and OKT8 markers, HLA-A and B histocompatibility antigens). At some stage of diseases like Down's syndrome, a compensatory increase in receptors, e.g. interferon receptors, has been described.[92]

Lymphocytes carry a large number of membrane proteins functioning as receptors for a whole variety of ligands (Table 1); these cells could therefore be considered as an easily available cellular system to investigate the state of hormonal and other physiological receptors. Some of these receptors appear to be located not on the cell surface but in the cytoplasm (e.g. corticosteroid receptors) while the presence of specific membrane receptors for some ligands which exert functional influences on lymphocytes (e.g. histamine) is still con-

Table 1 Lymphocyte receptors

1.	Antigen-specific : Ig, T-cell receptor
2.	Fc receptor
3.	C' components: C3a, C3b, C5a
4.	Lectins: PHA, Con A, PWM
5.	Histocompatibility antigens: DR, Ia
6.	Lymphokines: IL 2, interferon
7.	Hormones: glucocorticoids, insulin, growth hormone, beta-adrenergic, thymosin, calcitonin, parathyroid hormone
8.	Mediators: histamine, adenosine, acetylcholine, low-density lipoprotein

troversial.[93]

For insulin and corticosteroid receptors, an increase is observed during the early G1 phase and appears similar in the young and middle-age groups; the middle-age group, however, seems to lag behind in the late G1b phase, leading to an overall decrease in the number of receptors per activated cell at the end of the G1 phase. These results are consistent with previous publications indicating a decrease in sensitivity to hydrocortisone in aging lymphocyte populations.[94] For IL 2 receptors, we do not yet have indications whether the observed decrease in the number of IL 2 receptors occurs during the early G1a phase or only becomes manifest during the later phase of RNA synthesis. It is tempting to speculate that the basic aging phenomenon occurs at the level of RNA synthesis and/or transcription, preventing the synthesis and expression of several membrane proteins at a similar rate. This, however, would require more extensive investigations. As shown by experiments with serotonin,[84] the expression of IL 2 receptors and the production of IL 2 can be regulated independently. It would be interesting to assess whether age affects the RNA synthetic processes responsible for membrane receptor expression and lymphokine production to the same extent.

The changes in receptor expression and functions associated with age, at least in some other systems, do not seem to be entirely irreversible or to be impermeable to influence by exogenous factors. For example, dietary restrictions in middle age appear to affect favourably the rate of decay of glucagon and adrenergic receptors in adipocytes.[95] Similarly favourable effects of dietary restriction on the rate of decay of T-cell-dependent immune functions have been reported in old mice and rats.[96,97] *In vitro*, thymic hormones appear to modify the density of corticosteroid lymphocyte receptors.[98] Accordingly, it is tempting to speculate that some therapeutic regimens acting through effects related to receptor expression might reverse some of the functional immune defects observed with advancing age. Since one of the main apparent causes of impaired T-lymphocyte proliferation is a decrease in the number of PHA-activatable mature T cells and precursors[58] (possibly related to a decrease in the level of thymic hormones) and a deficiency in IL 2 production, it was logical to investigate whether concomitant administration of thymic hormones (in the form of an F5-like calf thymus peptides extract) and of IL 2 to old mice would restore their impaired T-cell proliferative capacity.[99] Preliminary experiments in our laboratory (F. Joncourt, unpublished results) suggest that such an approach is worth further consideration, although no spectacular reconstitutions have up to now been achieved, possibly because the defects in receptor expression are not influenced by the manipulations attempted.

Information on changes in lymphocyte receptors in human diseases has been rather scarce up to now. It is not unreasonable to postulate, however, that lymphocytes may reflect defects in receptor expression manifested in other cells as well. Such problems may in the foreseeable future be tackled at the individual cell level, which seems essential in order to permit the quantitative analysis of receptors on heterogenous cell populations. A combination of cytofluorometric analytical techniques using anti-receptor monoclonal antibodies should permit the establishment of "dynamic receptor profiles" under various clinical conditions. Since the density of many functional lymphocyte receptors apparently changes during the cell cycle, it appears essential to control the analytical conditions for this additional source of heterogeneity. The analysis of a receptor profile will therefore have to be dynamic not only in terms of following a disease's evolution but also in following the cells within their own cell cycle.

Summary

The combination of cytofluorographic analysis of human or murine lymphocytes proceeding through various phases of the cell cycle following lectin stimulation, the determination of DNA synthesis by ^{3}H-thymidine uptake, the quantitative assessment of lymphokines produced in culture supernatants (e.g. IL 2) and the evaluation of various cellular receptors (e.g. IL 2, insulin, corticosteroid) by fluorescent anti-receptor monoclonal antibodies or binding of radiolabelled ligand permits the development of a more comprehensive picture of the defects in lymphocyte functions which are associated with aging and various other clinical conditions.

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