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ABSTRACT

RECOGNITION REQUIREMENTS AND REGULATORY EVENTS DIRECTING T CELL RESPONSES.

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This study also indicated that ConA induced a very limited proliferation of Lyt-2$^-$ T helper cells (TH) in unselected T cell populations. The activation and growth requirements of Lyt-1$^+$ TH cells were directly investigated and compared with those of Lyt-2$^+$ cytotoxic T lymphocytes (CTL), as defined by the selective expression of Lyt differentiation antigens and functional activities. This analysis revealed a profound difference in activation and growth requirements between these T cell subsets. Thus, while Lyt-2$^+$ CTL precursors can be induced to TCGF reactivity by soluble lectins, in the absence of specialized accessory cells, Lyt-2$^-$ TH cell precursors show a strict accessory cell requirement both for activation and proliferation. Finally, the low level of TH cell effector function, detected in a primary responses to allo-MHC-antigens or lectins, appears to be due to the development of suppressive Lyt2$^+$ T cells.

The functional relevance of Lyt-2 antigens expressed on CTL membranes was further assessed in the last part of this study. Two distinct activation systems were used, namely MHC-antigens, provided as UV-irradiated stimulator cells or polyclonal induction by a 4 h pulse, with lectins. Both procedures were shown to selectively induce Lyt-2$^+$ CTL precursors into TCGF reactivity without leading to mitosis, unless TCGF was added. In both cases it was found that monoclonal anti-Lyt-2 antibodies inhibited the two antigen-dependent phases of CTL responses namely, the initial induction step and target cytolysis. The analogy observed between antigen specific and lectin mediated inducton and target cytolysis, with regard to the susceptibility of inhibition by anti-Lyt-2 antibodies has lead to a general hypothesis on CTL activation.

Key words: T cell growth factor production / Lyt-2-positive T suppressor cell/ Lyt-2-positive cytotoxic T lymphocytes / activation requirements of T lymphocytes / blocking of T cell functions.

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by

Martin Gullberg

CONTENTS

List of abbreviations:

Class I antigens	K and D region encoded antigens
Class II antigens	I region encoded antigens
ConA	Concanavalin A
CSF	Colony stimulating factors
CTL	Cytotoxic T lymphocyte
Ig	Immunoglobulin
LAF	Lymphocyte activating factor
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC (H-2)	Major histocompatibility complex
MLC	Mixed lymphocyte culture
TCGF	T cell growth factor
TRF	T cell replacing factor
TH	T helper lymphocyte
Ts	T suppressor lymphocyte
UV	Ultraviolet light

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This thesis is based on the following publications and manuscripts, which will be referred to by their Roman numerals:

I. Gullberg, M., Ivars, F., Coutinho, A., and Larsson, E.-L., Regulation of T cell growth factor production: Arrest of TCGF production after 18 h in normal lectin stimulated mouse spleen cell cultures. J. Immunol., 127:407, 1981.

II. Gullberg, M., and Larsson, E.-L., Studies on induction and effector functions of ConcanavalinA-induced suppressor cells abrogating TCGF production. J. Immunol., 128:746, 1982.

III. Gullberg, M., and Larsson, E.-L., ConA-induced TCGF reactivity is selectively acquired by Lyt-2 positive T cell precursors. Submitted for publication.

IV. Gullberg, M., Pobor, G., Bandeira, A., Larsson, E.-L., and Coutinho. A., Differential requirements for activation and growth of unprimed cytotoxic and helper T lymphocytes. Submitted for publication.

V. Gullberg, M., and Larsson, E.-L., Selective inhibition of antigen induced "step 1" in cytotoxic T lymphocytes by anti-Lyt-2 antibodies. Eur. J. Immunol., 12:1006, 1982.

VI. Gullberg, M. and Larsson, E.-L., Induction of cytolytic T lymphocytes by mitogenic lectins is specifically inhibited by anti-Lyt-2 antibodies. Submitted for publication.

I INTRODUCTION

1. The immune system

Ever since the appearance of replicating organisms on earth, there has been a continuous competition for "life space" between different forms of life. Later on, more differentiated creatures appeared which were not able to compete on the level of growth rates, and systems were developed to protect these from invasion of rapidly replicating environmental organisms such as viruses, bacteria, fungi and parasites. The most sophisticated defense system of this kind is the immune system of vertebrates.

The immune system consists of a large number of cells with different specialized functions. When an infectious agent succeeds in penetrating into the internal environment, the first attack is performed by granulocytes and macrophages, which constitute the unspecific part of the defense mechanism. If the invaders survive the first unspecific response, macrophages will present selected parts of their antigens to T and B lymphocytes, which are the cells responsible for the specific immune response. These cells resemble true aristocrates in the sense that most of them persist throughout their life span doing nothing. Nevertheless, if an antigen is present in sufficient amounts, clones which recognize it will be activated and start expanding. Thus, a large pool of lymphocytes are circulating in the lymphatic system and each expresses clonally distributed receptors. The different clones are, together, specific for all kinds of molecular patterns. If an antigen enters the system, only a minor fraction of this pool is able to specificially recognize its structure and initiate clonal expansion. This is the most fundamental concept in immunology and it was first visualized by Jerne in 1955 (1), and two years later modified by Burnet, as the clonal selection theory (2).

In mammals the immune system develops in the fetal liver but after

birth, bonemarrow is the major site for production of hematopoetic cells (3) lymphocytes included. All lymphocytes originate from a common pluripotential hematopoietic stem cell, which divide independently of antigen in the bone marrow and the progeny of which differentiates to specialized cells within the mammalian hematolymphoid system (4,5,6). During the differentiation in the bone marrow, B-lymphocytes precursors acquire immunoglobulins on the cell membrane. Immunoglobulins serve both as antigen specific receptors and effector molecules (7). As mentioned above, the lymphocyte compartment consists of a large number of cells and in the B cell system, each clone express immunoglobulin receptors with a given combining site or idiotype. According to the clonal selection theory, one clone is only allowed to express one type of combining site. In the light of the recent knowledge concerning the genetic organization of the immunoglobulin genes, it is possible to state that the requirement one clone - one specificy, is fulfilled (8-10).

In contrast to the B cells, maturation of the T cell lineage does not occur in the bonemarrow. Precommited T cell precursors enter the thymus from the blood stream and the intrathymic environment will give rise to an extensive proliferation. During this proliferative phase within the superficial thymic cortex the blast cell gives rise to a progeny of small lymphocytes (11). In the maturation process in the thymus the differentiating lymphocytes express specific determinants on their cell surface: Thy-1, Tla, Gv-1, Lyt and H-2 antigens (12). The mature T cells enter the peripheral lymphoid organs as small resting cells, expressing clonally distributed receptors.

The antibody diversity is probably generated in the bonemarrow while the repertoire of T cell specificities is generated during the intrathymic differentiation process (13-18).

Mature T cells can be divided into three major functional subgroups, namely T helper cells (TH-cells), cytotoxic T lymphocytes (CTL) and T suppressor cells (Ts-cells). Nothing is known with certainty either about the gene organisation or the biochemical structure of the

clonally distributed T cell receptor. Although some investigators have claimed that immunoglobulin idiotypes are present on T cells, recent molecular genetic studies have practically excluded participation of the immunoglobulin genes in the T cell receptor complex (19-21; L. Hood, J.F.A.P. Miller, S. Tonegawa. Personal communications, 1982). It is certain, however, that T cells recognize antigen in a different way than B cells. T cells appear to recognize antigens exclusively in association with the products of polymorphic genes at the major histocompatibility complex (MHC). It is possible, however, that there exists heterogeneity between different functional T cell subsets, also with regard to the antigen specific receptor.

T lymphocytes mediate the cellular immune response and they exert their effector functions either by the release of humoral factors or by mechanisms involving cell to cell contact, as is the case for CTL in the cytolysis of target cells. Also, TH cells trigger B cells by a direct interaction (22,23). Different T cell subsets can be distinguished from each other by the expression of certain cell surface markers belonging to the Lyt-system (24). Based on these differentiation antigens, T cells can be separated into three different subsets, namely Lyt-1^+, Lyt-23^+ and Lyt-1^+23^+ cells (25,26).

The TH cells belong to the Lyt-1^+ subset, which account for about one-third of all peripheric T cells, while Lyt-23^+ T cells constitute approximately 10-15 % of the T cell pool and include both the CTL and the Ts cells. The last subset, namely Lyt-1^+23^+ T cells, which accounts for approximately half of all T cells, seems to include all kinds of functional T cell activities depending on the inducing antigen (27,28). Despite the fact that exceptions (27,28) have been reported during the last years, the classification outlined above seems to remain useful.

Most immunologists believed for many years that antigens alone could activate lymphocytes and drive them into mitosis. Already 14 years ago, however, cell collaboration was demonstrated and, during the last 5 years a number of lymphocyte specific growth factors have been described, which participate in the cellular collaborative events and

are required for all types of immune responses (23,29-32). Lymphocyte growth factors are not specific for antigen, but apparently specific for each differentiative class of hematopoietic cells. Since immune responses are specific, however, there must exist a discriminatory event before the action of growth factors. This appears to be the case, since only lymphocytes which have recognized antigen specifically will be induced to express specific growth receptors (23,33). Thus, lymphocyte activation comprises two different steps, namely antigen recognition involving the clonally distributed receptors, and a cell type specific growth factor which drives the cell into mitosis. The initial triggering event will be further discussed in the next part of this introduction, while the third part will deal with production and action of lymphocyte growth and maturation factors.

2. Initial triggering of T lymphocytes

Mitchison (1954) and Lawrence (1959) suggested more than 20 years ago, that cell mediated immunity to bacterial antigens could only be created when these antigens were presented in association with self antigens of the individual (34,35).

Rosenthal and Shevach reported in 1973, that primed T cells could be restimulated to proliferate *in vitro* by adding the antigen together with syngeneic or semiallogeneic accessory cells, but not when adding it together with allogeneic cells (36). Furthermore, blocking of the T cell proliferation, was achieved by addition of antibodies directed to the MHC antigens present on the antigen presenting cell (36,37). Taken together, these observations suggested that T cells are activated only if the recognition of the antigen occurs in association with cell surface molecules encoded at the MHC complex. This phenomenon of obligatory recognition of antigen in association with MHC-encoded products has been termed MHC-restriciton, or in the mouse, H-2 restriction.

The MHC complex shows an enormous degree of polymorphism within any given species, and was first identified as being responsible for the phenomenon of graft rejection (38). The mouse MHC, the H-2 complex, consists of four major regions denominated as K, I, S and D region. H-2 K and D regions encode cell surface proteins present on all nucleated cells in the body, while H-2 I-encoded proteins are primarily expressed on B cells and antigen presenting accessory cells such as macrophages and dendritic cells. Some of the serum complement components are controlled by the H-2 S region (12).

Zinkernagel and Doherty found, in 1974, that virus specific CTL were able to lyse solely syngeneic virus infected target cells (39). Further analyses of this phenomenon have revealed that this restriction mapped to the K and D region of the H-2 complex (40). Miller et al in 1975 found that transfer of delayed type hypersensitivity caused by protein antigens, a function mediated by TH cells was H-2 I restricted (41). Despite some reported exceptions, there is a general agreement that antigen recognition by CTL is restricted to K and/or D-regions, while TH cells recognize antigen in connection with I-A or I-E regions encoded antigens (15,17,18,27,28,42-46).

The phenomenon of H-2 restriction of T cells is determined, prior to the challenge with antigen. Bevan (17) and Zinkernagel et al (15) have demonstrated, using F1 → parental chimeric mice, that the MHC-haplotype of the parent host governs the MHC restriction of the developing F1 T cells. The presence of MHC-encoded antigens on the epithelial cells of the thymus ensures that maturing T cells can learn "self preference" during the intrathymic steps of differentiation.

An important function, known to be controlled by genes located within MHC-region, is the control of specific immune response to distinct thymus-dependent antigens. These genes show the same degree of polymorphism as the MHC-region and has been termed "immune response genes" (Ir-genes) (47). It has been found that Ir-genes controlling I-region restricted responses map within the same region (48), whereas Ir-genes controling CTL responses have been located within the K and D regions

(16,49). These findings were the first indications that Ir-gene products and the antigens responsible for H-2 restriction are the same. Several mechanisms have been proposed to account for the influence that MHC antigens have on the response of T cells and most of these proposals can be classified into the three following broad categories: determinant selection that imply specific association of antigen and MHC molecules to present unique antigenic determinants (50,51), clonal deletions of self reactive T cell clones that will give rise to a hole in the T cell repertoire (52) and finally a MHC specific T cell selection process based on a degree of reactivity to self-MHC-encoded molecules expressed in the thymus (14,16,53-55). The mechanisms proposed above are not mutually exclusive and provide explanations for most experimental observations to date.

The theory of Jerne on the generation of diversity (14), later modified to include restricted T cells (16), accounts for these phenomena, namely Ir-gene effects, MHC-restriction and MHC-dependent allo-reactivity. The basic postulates are a complete germ-line repertoire of T cells for MHC determinants of the species and dual specificity of T cells for MHC-determinants and antigens. The details of the "dual specificity" shown by restricted T cells, however, have been much debated. Von Boehmer et al (16) proposed a two receptor model which postulate that a T cell has two different receptors, one for antigen X, and another for self MHC antigens (Fig. 1). One of the problems of all dual receptor models, are the results from cold target inhibition experiments. Inhibition with unlabeled target cells is only obtained when the restricting element and the antigen are presented together on the same cell surface (56). In the competing model, generally refered to as the altered self hypothesis, it is assumed that an antigen X associates with the MHC antigen to form a hybrid molecule, or a new-antigenic determinant, and this complex is then recognized by a single receptor (57,58). Since all T cells are reactive to modified self MHC antigens, it is likely that they crossreact with allo-MHC-encoded antigen, explaining the high frequencies of alloreactive T cells (59). This hypothesis is supported by observations that H-2 restricted killer cells specific for antigen X, can lyse allogeneic target cells

expressing neither the antigen X, nor the restricting self-MHC-encoded antigen (17,60-62).

Personally, I regard the altered-self hypothesis as an attractive model because of its simplicity and the demonstration that T cells, which are specifically reactive to allo-MHC-encoded antigens, can indeed recognize foreign antigens in an H-2 restricted manner.

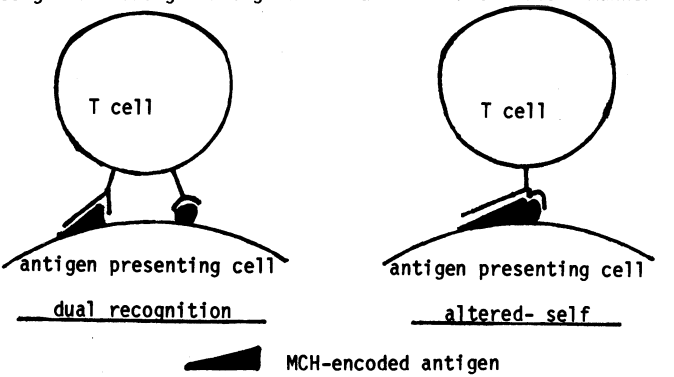

Figure 1.

A vertebrate contains between 10^8-10^{12} lymphocytes with various specificites. According to the concept of clonal selection theory, only a few clones will be activated and expanded in an immune response, while all others are kept resting. The study of lymphocyte activation constitutes, therefore, a fundamental aspect of immunology. In the study of a specific immune response, defined antigenic structures like haptens or synthetical copolymers serve as important tools. These antigens however, give rise to clonal responses which are too small to be studied in unprimed lymphocyte populations, from cell biological and biochemical points of view.

Polyclonal activators, also termed mitogens, have been very useful in the study of lymphocyte activation since they overcome this difficulty and stimulate large numbers of cells. Lectins constitute a large group among these mitogens and they have been extensively used in different experimental systems (63-65). These proteins, with exquisite

specificity for sugar residues on cell surface glycoproteins, bind to abundantly represented target proteins on all lymphocyte cell membranes (66). Mitogenic lectins however, selectively induce T cells to proliferate, demonstrating the specificity of activation and the difference from binding. The increase in immunoglobulin synthesis observed under some conditions is a consequence of collaborative interactions with lectin-activated TH cells (67-69).

The demonstration that lectin-mediated T cell activation, as antigen-induced responses, requires the participation of accessory cells, has underlined their value as a model system for studying the mechanisms of cell cooperation involved in T cell triggering (70-73). Recent reports have demonstrated that lectin induced proliferation is a result of an intricate team work between different cellular subsets, which allows for the conclusion that mitogenic lectins are in fact no true mitogens (32,33,73,74). Furthermore, only few of all the lectins, known to specifically recognize sugar determinants on T cells, are polyclonal T cell activators (66), again showing the difference between binding and triggering.

It is still not established whether lectin mediated activation of T cells is a true immunological reaction, i.e., employing clonally distributed receptors, or if lectins bypass the requirement of specific recognition by interacting with a hypothetical "activation site". In view of the amount of studies performed with mitogenic lectins, it is of importance to find similarities or differences between lectin and antigen induced lymphocyte activation.

3. Lymphocyte growth factors

As previously outlined, lymphocyte growth is dependent on the hormone-like action of growth factors. Already in 1965, soon after the discovery of polyclonally activating lectins, soluble mitogenic factors were found in supernatants of lectin stimulated cultures (75,76). For

many years, many different kinds of biological effects were attributed to these factors and a rather confusing picture of their biological significance emerged. A real breakthrough occured in this field as Morgan et al (77) observed that conditioned media from lectin stimulated lymphocyte cultures contained growth promoting activities for bonemarrow T cells in vitro. These findings were soon expanded by Gillis and Smith (78), who showed that the same principles could be applied to specifically activated T cells, making it possible to propagate and expand activated T cells, and offering the way to the recent development of in vitro studies on cloned T cell populations (78-83). The protein responsible for the growth promoting activity for T cells has been characterized as a glycoprotein with a molecular weight of 15 K in human and 30 K in mouse (29).

Supernatants of lectin stimulated lymphocyte cultures however, do not only contain T cell growth factor (TCGF) but also a multiplicity of activities affecting, for instance, B cells (T cell replacing factor (TRF))(84), hematopoietic stem cells (colony stimulating factors (CSFs)), and several other factors activating macrophages and mast cells (85-87). The list of activities can be made very long and it would seem that there exists as many factors in such a supernatant as there are assays described for lymphokine activities.

The nonspecific action of TCGF itself, indicated that the process of T cell activation could not simply be the generation of growth factors, as immune responses are specific. The discovery that TCGF was non-mitogenic on small unprimed T cells made it possible to introduce an initial discriminatory step, involving clonally distributed receptors, which induces TCGF responsiveness (33,88,89). This model of T cell activation was demonstrated by the finding, that 4-5 h pulse with lectin or antigen results in induction of TCGF reactivity in an unprimed T cell population, which is otherwise, resistant to the mitogenic activity of the factor. Induced cells are subsequently able to specifically absorb the growth promoting activity (33,90). Furthermore, Robb et al have recently demonstrated, by using a radiolabeled

homogeneous TCGF preparation, that induction of TCGF reactivity gives rise to more than a 1000-fold increase in TCGF binding sites on T cells (91).

The lectin-dependent induction of resting T cells to TCGF reactivity does not require accessory cells, while production of growth factors is a cell cooperative response which involves TH cells and I-A$^+$ macrophages in conjunction with the lectin (32,33). The subsequent TCGF dependent proliferation takes place independently of the lectin and of any other accessory cell (92). I-A$^+$ macrophages can be substituted for by a differentiation factor (lymphocyte activation factor, LAF) which enables purified T cells to produce TCGF (32). LAF is produced by I-A$^+$ macrophages, the induction of which is the T cell dependent responses of nonprimed populations requires a TH cell, distinct from the TCGF producing T cell and designated T "inducer" cell (74).

The model of T cell activation, which we have proposed, is schematically presented in Fig. 2 (93).

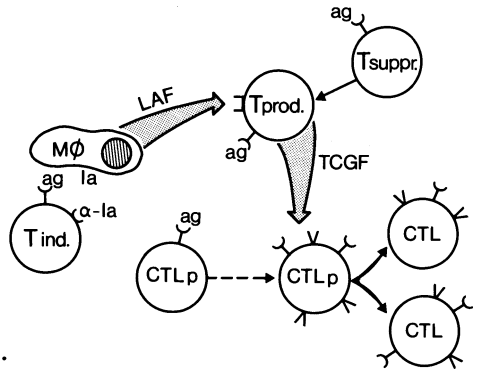

Figure 2.

Several investigators have recently described a B cell specific growth factor with similar biological properties as TCGF (23,30,31,94,95). Furthermore, evidence for the existence of different kinds of maturation and differentiation factors acting on CTL or B cells was recently obtained (84,96,97). Even B cell proliferation induced with

lipopolysaccharide (LPS), which has been believed to be a "true" mitogenic process, appears to depend on factors produced by macrophages (98).

Personally I believe, that it is now the time to leave "the period of crude supernatants" in favour of well defined homogenous protein preparations, or we will soon be back to the same type of confusions we thought we left some years ago.

II OUTLINE OF THE PRESENT STUDY

The broad aims of this investigation have been to study the cellular and molecular requirements in T cell responses. The regulation of TCGF production appeared to be of particular interest, since the availability of this growth factor seems to be the only limiting variable for the clonal expansion of TCGF- reactive cells. The initial part of the work presented here is concerned with the analysis of the regulatory events directing TCGF production by normal spleen cells stimulated with Concanavalin A (ConA). These studies focused our interest on the growth and stimulation requirements of different T cell subsets. The cellular subsets were defined both functionally and by the selective expression of Lyt differentiation antigens. The functional relevance of Lyt-2 antigens, which are present on CTL membranes, was further assessed in the last part of this study. This approach also resulted in a comparative study of the mechanisms underlying the activating properties of allo-MHC-antigens and polyclonally activating lectins.

1. Regulation of T cell growth factor production

The peculiar kinetics of accumulation of TCGF activity in ConA stimulated cultures first attracted our attention. Thus, a study was performed to evaluate the mechanisms regulating TCGF production in these cultures. It was found that normal spleen cells, activated by ConA for 24 h, develop suppressive cells that were able to block *de novo* TCGF production by fresh spleen cells. The effector cells mediating suppression of TCGF production have been characterized as nonadherent, Lyt-2-positive, T cells. The induction time for these suppressor cells, in primary cultures, was found to be 18 h and to very closely parallel the termination of TCGF production *in situ*. Furthermore, incubation of 24 h ConA-activated cells, in the absence of

the lectin during a 24-72 h period, leads to a gradual loss of suppressor activity. This activity however, could be reinduced by re-addition of ConA, with the same kinetics of appearance as in primary conditions. The ConA-activated cells fully recovered their capacity to produce TCGF after a lectin free culture period of 72 h, showing that TCGF responsive cells were still present. Taken together with the fact that no correlation was found between suppressive and cytolytic activtity, these results show that inhibition of recovery of TCGF in these supernatants is neither due to *in situ* absorption of TCGF produced at control rates nor to killing of TCGF producing T cells.

2. Growth and stimulatory requirements for different T cell populations.

It is now generally accepted, that TCGF conditioned medium is sufficient to maintain CTL lines or clones *in vitro* for a prolonged period of time. In contrast, however, the stimulation requirements for TH cells are still controversial and most of the present studies has been performed on TH cell lines or clones, maintained *in vitro* for a prolonged period of time.

The present study is concerned with the activation and growth requirement of CTL and TH cells in an unprimed purified T cell population. As markers for these subpopulations, both the expression of Lyt differentiation antigens and functional assays for various effector cell activities have been used. The experimental findings can be summarized as follows. A profound difference exist between CTL and TH cell precursors in their activation and growth requirements. Thus, while Lyt-2^+ CTL precursors can be induced to TCGF reactivity by soluble lectin in the absence of adherent accessory cells, Lyt-2^- TH cell precursors show a strict accessory cell requirement for activation and proliferation. Furthermore, exogeneously added TCGF has no effect on TH cell proliferation and effector functions, in contrast to CTL proliferation, which is exclusively dependent on this growth factor.

Finally, the low levels of effector helper functions, detected in a primary response to allo-MHC-antigens or lectins, appears to be due to the development of suppressive Lyt-2^+ T cells. Thus, in order to study TH cell growth and differentiation to effector cells, Lyt-2^+ T cells must be removed from an unselected T cell population.

3. Recognition requirements involved in induction and target cell lysis of cytotoxic T lymphocytes.

The last part of the present investigation deals with the effect of monoclonal antibodies (mAb) against cell surface molecules at various levels of CTL induction, growth and effector functions. All the mAb used are of the same Ig class and specific for T cell differentiation antigens, expressed in equivalent amounts, such that specific effects can be evaluated. In this approch, two distinct systems of activation were used, namely allo MHC Class I antigens provided as UV-irradiated allogeneic stimulator cells or a 4 h pulse with a polyclonally activating lectin. Both procedures were shown to selectively induce Lyt-2^+ CTL precursors to TCGF responsiveness without leading to mitosis, unless exogeneous TCGF is added to the system. Thus, these systems makes it possible to selectively study initial antigen recognition, interjacent TCGF dependent clonal expansion and target cell cytolysis by Lyt-2^+ CTL. In contrast to anti-Thy-1.2 and anti-Lyt-1.2 mAb, a profound inhibition was exerted by anti-Lyt-2.2 mAb on both CTL functions which involve antigen recognition, namely the initial induction step and target cell cytolysis. The interjacent TCGF dependent proliferation, however, was resistant to the same mAb. The parallelism observed in these protocols between antigen-specific and lectin-mediated activation or target cell cytolysis, in regard to the susceptibility to inhibition by anti-Lyt-2.2 mAb, lead to a general hypothesis on CTL activation. It is assumed that Lyt-2 antigens are functionally associated with specific recognition receptors on CTL and

since lectin-dependent induction is also sensitive to Lyt-2 blocking, it is postulated that lectins activate CTL upon binding to this receptor complex as well. Since lectins, however, bind to many other surface glycoproteins on T cells, but only the Lyt-2 associated sites are triggering complexes, it is concluded that CTL can only be induced by interactions with specific clonally distributed receptors. It would follow that "mitogenic" lectins bind to clonally distributed receptors on CTL and that such experimental tools may be of value in investigations of T cell repertoires and MHC restriciton.

III GENERAL DISCUSSION

In the following sections I have attempted to summarize and discuss my experimental findings in general terms. In addition, a brief overview is given for each part of the present work.

Since the publications and manuscripts, on which the thesis is based, already contain the detailed experimental results and separate discussions, there is no reason for me to repeat them here. Rather, I will especially concentrate on the more controversial parts of this work, where conflicting results have been reported, or different opinions exist at present. I think that it will be quite obvious for the non-immunologists that immunology can not be noted for its lack of controversies.

1. Negative regulation in a T cell response

Regulation of immune responses is often thought to be due to the activity of suppressor T cells. Among T cells, however, the Ts subgroup is the most controversial. These cells have been attributed effector functions in both cell mediated and humoral immune responses. Various kinds of Ts cells, which differ in specificity and mechanisms of action have been described in the literature, for instance; allotype specific Ts cells (99), idiotype specific Ts cells (100,101), carrier protein specific Ts cells (102) tumor specific Ts cells (103,104), and Ts cells inhibiting delayed type hypersensitivity responses (105,106).

Many immunologists are very critical in regard to the interpretation of experiments with Ts cells and even to accept their existance as a defined member of the immune system. One of the reasons for this is that an immune response that has failed to appear, can always be explained in many different ways. Thus, it is an inherent characteristic in most systems measuring suppression, that it is almost

impossible to include all the appropriate controls. Another serious problem in the T suppressor field is that the only cellular marker defining the Ts cell population, namely the I-J encoded determinant, has very recently been questioned.

Steinmetz et al (107) have shown, by using overlapping cloned genomic DNA sequences, that the I-J subregion is confined to a 3,4 kb region of DNA, that is far too little to encode the traits of the I-J region. From these molecular genetic results, it could be concluded that the I-J subregion may be a genetic artifact. It should be noted in this context, that ever since the original description of the I-J region by Murphy et al (99), putative I-J specific antisera have been extensively used in Ts studies (108). It is remarkable, however, that no gene product has been isolated and characterized, despite the fact that cell lines and clones, expressing I-J determinants, have been reported (109,110), as well as monoclonal antibodies with this specificity (111,112).

Despite the problems outlined above, very few immunologists today dispute the existence of a T cell population that is able to block other T cell functions. It is still a matter of controversy, however, whether or not these functions are mediated by a specialized T cell subpopulation, and the overall importance of this kind of immune regulation remains an open question. Furthermore, the cellular markers that have been reported for Ts cells are also present on CTL or TH cells, except the elusive I-J determinant.

As in many other cases, also for Ts cells mitogenic lectins have been explored to activate effector functions. In several systems, ConA activated T cells have been shown to suppress mitogenic and antigen specific responses (113,114). Furthermore, spleen cells from mice injected with ConA in vivo are markedly suppressed in their capacity to be restimulated in vitro with ConA or PHA (115,116).

In the present study, the regulatory mechanisms directing TCGF production in a ConA driven bulk culture, were investigated (paper

I,II). We have found that TCGF production in ConA stimulated cultures is arrested 18-24 h after initiation. Cell transfer experiments indicated that suppressive cells, able to block de novo TCGF production, were induced in such cultures. Effector cells mediating suppression were characterized as non adherent, radioresistant, Lyt-2-positive T cells. The induction phase of these cells is radiosensitive and requires 18 h of culture with the lectin.

2. Possible mechanisms of action of T cells capable of blocking TCGF production.

Several investigators have proposed that Ts cells exert their effector function by secreting soluble factors. Many of these suppressor factors have been characterized as antigen specific, idiotype and I-J positive (103,117,118). As we are very critical towards the concept of antigen specific factors in general, and as the existence of I-J determinants is highly questionable, we do not consider these factors to constitute an attractive general mechanism of suppression.

A soluble inhibitor that suppresses the proliferative response of T cells to alloantigen have been found in MLC supernatants. This factor, with an apparent MW of 10.000, was found to suppress the production but not the function of TCGF (119). Also, a substance has been found in sera from normal mice, that inhibit the function of TCGF (120). Our attempts to block TCGF production with culture supernatants from suppressed cultures, however, have been without success. Although some inhibition was observed, it was not at all comparable with the potent suppressive effect exerted by 24 h ConA activated cells.

Another plausible mechanism of suppression is lectin facilitated killing of TCGF producing TH cells in culture, by activated CTLs. This, however, is unlikely because 18 h of ConA activation is not sufficient to induce efficient cytotoxic activity by CTLs and no

correlation was found between cytotoxic and suppressive activity. Others have also shown that suppression in MLC systems by ConA or antigen activated suppressive cells, is not due to cytotoxic activity (121,122).

Larsson et al have shown that a 4 h pulse with ConA is sufficient to render T cells capable of absorbing TCGF activity (33). Furthermore, it has been demonstrated that a TCGF dependent CTL clone can suppress the generation of cytolytic activity in MLC cultures, presumably by absorbing TCGF (123). It is clear, therefore, that the suppression of recovered TCGF activity in supernatants exerted by ConA activated T cells can, at least partly, be attributed to absorption. We do not accept, however, that absorption of TCGF produced at normal rates is the sole mechanism of suppression as proposed by Palacios et al (124). The evidence supporting some other type of suppression mechanism is the following:

1. The number of activated T cells necessary to remove TCGF, in an amount equivalent to that present in a 24 h ConA supernatant, is hundred fold higher than the number of irradiated Ts cells able to abrogate TCGF production (38,89).

2. The assumption by Palacios et al is based on the finding that the proliferative response, in a mixed culture of fresh and ConA activated cells, is restored by addition of preformed TCGF. However, this evidence is irrelevant as the same result could be expected regardless of the mechanism(s) behind suppression of TCGF production. Furthermore, it was reported that addition of the immuno-suppressive drug cyclosporin A to the primary ConA culture abrogates induction of Ts cells. From our point of view, this could also be expected, since it confirms our observations that DNA replication is required for induction of effector Ts cells (II).

3. Since ConA activated CTLs absorb TCGF very efficiently, there should be a clear correlation between suppressive and cytotoxic

activity, and this is not the case (II).

4. The maintenance of suppressive activity required the continuous presence of ConA, and we have shown that Ts cell activity reverts within a period of 48 h to 72 h in the absence of lectin. These cells, however, still proliferate in response to TCGF, and thus must be competent to absorb TCGF. Even more directly, Kumagai et al have reported that spleen cells deprived of ConA retain functional TCGF receptors for at least 72 h (125).

5. The induction time for Ts cell activity is between 18-24 h and parallels very closely the termination of TCGF production in situ, whereas it has been demonstrated that a 4 h pulse with ConA is sufficient to activate in spleen cells competence to absorb TCGF (33).

Taken together, our observations suggest that suppression of TCGF production is an active process mediated by Lyt-2^+ T cells, which directly and reversibly block TCGF producing cells. Such reversibility excludes killing of producer cells, and the precise mechanism of inhibition still remains unknown. There are some indications that cell-to-cell contact is required, since the suppressive activity on a per cell basis decreases with the cell density in test culture. The total TCGF production in control cultures, however, decreses drastically with the cell density and these experiments are consequently difficult to perform in a low cell density culture.

Nevertheless, recent results extend these findings and give them larger biological perspectives. As discussed below, it is clear that ConA induces very limited proliferation of Lyt-2^- TCGF producing cells in an unselected splenic T cell population (IV). Interestingly, however, removal of Lyt-2^+ cell from such a population enables proliferation and enrichment of Lyt-2^- TCGF producing cells. Thus, ConA per se is able to induce Lyt-2^- cells to proliferation and effector functions, but it seems that the simultaneous activation of Lyt-2^+ suppressive cells blocks various types of TH cell functions, in addition to result in

preferential growth of Lyt-2^+ cells in cultures of unselected cells.

Other phenomena of ConA induced suppression of T cell functions that might be related to this discussion have also been observed in limiting dilution type of experiments. Thus, an unfractionated spleen cell population contains cells able to inhibit growth of a large fraction of reactive clones stimulated with ConA and TCGF (126). Eichmann et al have observed a similar phenomenon of suppression in frequency determinations of streptococcus A specific TH cells, in ConA induced splenic T cell populations (127).

3. The target cell for TCGF

The original finding by Gillis and Smith (78) that tumor specific CTL could be enriched and maintained in the presence of ConA conditioned media, indicated that CTLs were the targets for the growth promoting activity contained in those supernatants, which was then defined as TCGF. It was later reported that TH clones could also be maintained and retained their specific effector function, in the presence of preformed TCGF and in the absence of antigen and accessory cells (128,129). In contrast, however, Larsson et al (130,131) found that the growth of TH cells, enriched by an alternative method, was not supported by TCGF, but that continuous antigen recognition on competent stimulator cells was required for the maintenance of specific helper function. Most likely, these conflicting reports reflect how various in vitro selection and enrichment techniques can give rise to T cells with different growth requirements. It seems that exogenously supplied TCGF, at the beginning of enrichment cultures for TH cells, results in cells that are strictly dependent for growth on TCGF or some other unknown factor present in the TCGF containing preparation used.

At present it is hard to judge whether it is the TCGF-dependent or TCGF-independent TH cell which is the "physiological" type of helper cells. Both types of TH cells have been shown to be active in vivo and reconstitute T dependent responses in nude mice (132,133). Furthermore, it has been reported that some TH clones release TCGF upon antigenic

stimulation and, therefore may be growth supported by their "own" TCGF (129,134,135). The major problem in this context is the lack of homogeneous preparations of mouse-derived TCGF. With the TCGF preparations available today, it is impossible to state whether the same, or distinct, molecular entities promote both TH cell and CTL proliferation.

In most studies concerning TH cell growth, extensively primed TH cells or clones enriched and maintained *in vitro* for prolonged periods of time have been used. Obviously, these studies may produce conflicting results due to the various *in vitro* selection protocols. The discrepencies outlined above make it important to investigate activation and growth requirements for various T cell subpopulations in unprimed populations. The experiments described in paper III, IV, V and VI demonstrate, that after initial activation, TCGF dependent growth is almost exclusively acquired by Lyt-2^+ CTL precursors. The evidence for this conclusion is the following:

1. A Lyt-2^- T cell population is not able to initiate TCGF dependent exponential growth after a 4 h pulse with ConA (III). Furthermore, limiting dilution experiments reveal that the frequency of ConA inducible TCGF reactive clones, is 99 percent decreased if Lyt-2^+ cells are removed from an unselected T cell population (III).

2. UV-irradiated allogeneic cells, which are nonstimulatory in conventional MLC, selectively induce Lyt-2^+ cells to initiate TCGF dependent growth (V). Under these conditions, solely antigen triggered TCGF dependent cells are able to proliferate and mature, since UV-irradiation, or glutaraldehyde fixation, of stimulator cells does not induce TH cell functions in primary cultures (V,136,137).

3. T cell populations obtained by initial activation with ConA or UV-irradiated stimulator cells contain, after 5 days of TCGF dependent proliferation, a cytotoxic potential comparable to a cloned CTL line. Such a TCGF expanded T cell population is, on a

per cell basis, about 5 times more efficient in target cell lysis, as compared to effector cells generated in a conventional MLC. These results strongly indicate a homogeneous CTL response and therefore, a strong selection for CTL growth by TCGF.

The results outlined above are also supported by the previous demonstration by Larsson et al (138,139) that all TCGF reactive clones, derived after polyclonal activation of splenic T cells with either ConA or LA, are able to kill tumor targets in the presence of PHA. The high frequency of ConA-inducible TCGF-reactive cells obtained in these protocols (1 in 5) (III,138,139), indicates that both the Lyt-1$^-$23$^+$ and Lyt-1$^+$23$^+$ subsets are induced to TCGF reactivity, as previously suggested by others (138,140,141). The same is also shown by the decreased TCGF dependent proliferation observed in negatively selected Lyt-1$^-$23$^+$ cell populations, as compared to the response obtained with unselected T cell populations (III). The phenotype Lyt-1$^-$23$^-$ is used in this discussion on operational basis only, to indicate T cell populations remaining after treatment with monoclonal anti-Lyt-1 antibodies and complement. I am aware that the Lyt-1 marker appears to be expressed on all T cells (142), but the cytotoxicity data would indicate large differences in the concentration of this marker on different sets of T cells.

4. Growth requirements for Lyt-2$^-$ T helper cell precursors

In the previous section, the TCGF reactive cell in primary responses was characterized as a Lyt-2$^+$ CTL precursor, and the results indicate that CTLs and TH cells are not only functionally distinct but also that they have differential requirements for activation and growth. It is generally accepted that Ia positive macrophages play an essential role in antigen presentation (36,143-147), but as recently shown and confirmed in this investigation, it appears that ConA induced acquisition of TCGF reactivity is an accessory cell independent event (V,32,33). On the other hand, as outlined in the previous section, the

requirements for antigen carrying macrophages and growth factors for TH cell lines or clones, appears to be directed by the strategies used in the initial selection and enrichment phase. Thus, the conflicting results, in regard to stimulation and growth requirements of TH cell lines, stress the importance of studying the cellular and molecular requirements for a primary response of T cells with helper functions.

The present study contains an initial attempt to establish the requirements for primary activation and growth of different T cell subsets. For defining these subsets, we have used both the selective expression of Lyt differentiation antigens and, as functional markers, various assays for detection of effector functions. Due to the difficulties in measuring growth in a primary antigen specific response, ConA was used as inducing ligand and consequently, we have used assays for functional activities of effector cells, which overcome clonal recognition. The main conclusions from this study can be summarized as follows (IV):

1. In contrast to unselected T cell populations, Lyt-2$^-$ T cells are not induced to TCGF reactivity and require the continuous presence of the lectin and adherent accessory cells for proliferation.

2. Partially purified TCGF added to cultures containing Lyt-2$^-$ T cells stimulated with ConA and macrophages does not have any significant stimulatory activity. Furthermore, the functional activities of these cells are unaltered in the presence of TCGF.

3. The growth characteristics were the same for TH cells mediating help to CTL (by TCGF production) and TH cells that activate B cells to proliferation and terminal maturation. No effector CTL, however, developed in these cultures.

4. Development of effector helper functions was totally inhibited by the presence of Lyt-2$^+$ T cells.

5. Analysis of the expression of Lyt-1 and Lyt-2 antigen suggests that the induction of Lyt-1-23$^+$ TH cells is inhibited by the presence of Lyt-2$^+$ T cells.

6. In ConA-induced responses, the Lyt-phenotype of responder cells is well correlated with cytotoxic versus helper activities.

7. Finally, effector helper function is increased more than ten-fold in a Lyt-2$^-$ T cell population, after 5 days in the presence of ConA and macrophages. This is to our knowledge, the first demonstration that TH cells can be stimulated to proliferation in a primary response to ConA.

Although the experiments outlined above do not address the question of the Lyt-2$^-$ TH cell specific growth factors, they demonstrate a marked contrast between CTLs and TH cells in primary activation requirements. Thus, even if it is not possible to totally exclude a role of accessory cells in the induction of TCGF reactivity, there is a remarkable quantitative difference between CTL and TH cells in macrophage dependence for activation and growth. It has previously been shown that TH cells are able to produce TCGF, in the absence of accessory cells, if LAF is present in culture (33,148). It must be pointed out, however, that TCGF production can occur without cellular proliferation (II), and preliminary results indicate that LAF prepared from the P388D macrophage line, can not reconstitute macrophage dependent proliferation of unprimed Lyt-2$^-$ TH cells or extensively primed TH cell clones (Gullberg and Pobor, unpublished observations). Thus, it seems that LAF is required as a signal for TCGF production, but does not drive TH cells through the mitotic cycle.

In a recent study, Bandeira et al have shown that MHC restricted TH cell clones, specific for "minor" antigens expressed on B cells and macrophages, were strictly dependent on adherent macrophages for cellular proliferation (98). In contrast, although carrying the same antigens and "restricting" I-region products, B cells were totally unable to stimulate TH cell proliferation. However, these TH cells were

able to activate B cells, carrying the specific antigens, in the absence of macrophages and thus, proliferation was not required for helper functions. These observations closely parallel the results obtained with unprimed TH cells, in that proliferation and helper function appear to be functionally separated. Thus, macrophages seem to be required for TH cell growth but effector helper function can occur in the absence of proliferation, if a nonmitotic signal, such as LAF or antigen carrying B cells, is present.

In the present study, we have concluded that the clonal expansion of Lyt-2^+ CTLs are strictly dependent on TCGF produced by TH cells, in contrast to Lyt-2^- TH cells which require antigen presenting cells for their growth. Very recently, however, Lyt-2^+ CTL clones have been isolated, that are able to proliferate in the absence of exogeneously supplied TCGF, if appropriate stimulator cells are present (149,150). These CTL clones have the same cytotoxic potential as the TCGF dependent ones, but they resemble "hybrids" between a TH cell and CTL in the sense that they appear to produce their own growth factor(s). The "antigen driven" CTL clones kill the appropriate tumor cell if injected into a normal mice, but the relative importance of this type of cells still remains to be established.

Finally, I want to stress that, although cloned populations of TH cells have not provided conclusive answers concerning their own growth requirements, they have been extremely useful in the study of T-B collaboration and the analysis of the fine specificity of T cell recognition.

5. Functional significance of the Lyt series of differentiation antigens.

Differentiation antigens of the Lyt series are widely used to dissect interactions involved in T cell regulatory and effector functions. As a result of the original work of Cantor and Boyse (24,25), it became

generally accepted that TH cells express the Lyt-1$^+$23$^-$ phenotype, whereas CTLs are included in the Lyt-1$^-$23$^+$ subset. However, Ledbetter et al (142) have later demonstrated, that the Lyt-1 marker is present on all cells of the heterogenous peripheral T cell population, but in a wide range of concentrations, from high to low expression. Several groups have recently confirmed these findings and they have been able to demonstrate, by treating cell populations with high titers of anti-Lyt-1 antibodies and complement, that the majority of CTL precursors exhibit the Lyt-1$^+$23$^+$ phenotype (140,141). Furthermore, it has been proposed that the Lyt phenotype of T cells may correlate more directly to their restriction to Class I or Class II MHC antigens, than to their subclass or effector function (27,28,45,151).

In the present study we have used anti-Lyt-1 and anti-Lyt-2 mAb's, in order to separate TCGF responsive cells from the unresponsive population (III). As discussed in the previous sections, it is quite clear that Lyt-2$^-$ cells are unable to initiate antigen independent proliferation in the presence of TCGF. The significant reduction observed in a Lyt-1$^-$ T cell population remains, however, to be clarified. It should be noted in the present experiments, that the same cytotoxic activity of each monoclonal antibody as tested on thymocytes, was used in the complement mediated depletion of the respective T cell subgroups. It would appear, therefore, that the Lyt-1 antigen is expressed on all CTL precursors, but some of these cells do not express sufficient amounts of the protein to be lysed by the cytotoxic treatment used in the present experiments. Nevertheless, the TCGF production within the Lyt-1$^-$ subset, or rather within the population expressing low amounts of Lyt-1, was quite decreased as compared to the amounts produced by the Lyt-2$^-$ T cell subset. Thus, it seems that the Lyt-1 antigen is no longer useful as a qualitative marker, but remains useful in quantitative terms, to distinguish functionally distinct T cell subpopulations.

The Lyt-2 protein, on the other hand is still a respectable cellular marker for CTL despite some recent controversies (152,153), and some examples of Lyt-2$^+$ cells with helper functions which have been

reported in the literature (45,151). In marked contrast to the Lyt-1 antigen, that is expressed on all T cells in a wide gradient of concentrations, the expression of the Lyt-2 marker defines a quite distinct population. The Lyt-2 antigen is always co-expressed with the Lyt-3 antigen, and Ledbetter et al (154) have shown that the Lyt-2 and Lyt-3 proteins constitute a complex of disulfide-bonded subunits.

Cell mediated cytotoxicity is generally resistant to treatment with antibodies specific for various cell surface determinants. The original finding by Shinohara and Sachs (155), namely that antibodies specific for Lyt-2 antigens were capable of blocking specific killing, called much attention to the Lyt-2 protein. Numerous reports describing this phenomenon have appeared during the last 3 years (142,156-160). Many investigators have speculated that the Lyt-2 protein could constitute a constant part of the "elusive" T cell receptors. These speculations were further encouraged by the finding that light chain markers are encoded by genes that are tightly linked to the Lyt-2,3 locus (161,162).

6. Blocking of antigen recognition by a monoclonal anti-Lyt-2.2 antibody

The accumulating evidence, suggesting that the Lyt-2 protein is involved in CTL mediated cytotoxicity, promoted us to perform a detailed study to probe the effect of an anti-Lyt-2.2 mAb, at the various levels of T cell functions. It was of particular interest to investigate whether this mAb was able to selectively block Lyt-2^+ CTL in the two events known to require antigen recognition, namely the initial induction to TCGF reactivity and cytolysis of the specific target cell. In an approach to evaluate the functional role of the Lyt-2 antigen, it is essential to use an antigenic system that selectively induce Lyt-2^+ CTL. Previous reports concerning the effect of anti-Lyt-2 antibodies added to a conventional MLC, had not reached clearcut conclusions (163), since a high frequency of Lyt-2^- clones are activated to proliferate (V,24,141) and a multiplicity of cell

interactions are involved in the overall responses detected in such a system. Thus, for the reasons discussed above, we have defined an antigen specific system, where induction to TCGF responsiveness, TCGF dependent proliferation and effector CTL function can be studied in separate. As mentioned in a previous section, exposure of T cells to UV-irradiated allogeneic stimulator cells, does not result in a cytolytic response. It had also been shown, however, that both the proliferative and the cytolytic responses can be restored by supernatants obtained from a secondary MLC (V,136,137).

We have characterized this system (MLCuv) and found that:

1. TCGF (but not LAF) is competent to reconstitute the proliferative and cytolytic response.

2. Upon reconstitution with TCGF, five times higher cytolytic activity with a low range of crossreactivity, is obtained on a per cell basis from MLCuv, as compared with a conventional MLC system.

3. No in situ TCGF production is detected in MLCuv and all responding cells in TCGF reconstituted culture carry Lyt-2 antigens.

We conclude from these findings that UV-irradiated allogeneic cells, selectively induce specific Lyt-2^+ CTL precursors to acquire TCGF responsiveness.

These characteristics of MLCuv, make it an attractive system to use in our attempts to probe the effect of Lyt-2 antigen blocking on responder T cells, and to evaluate specific inhibitory effects on antigen-dependent induction of TCGF reactivity. As shown in paper V, precoating of responder T cells with anti-Lyt-2.2 mAb, results in a profound inhibition of the acquistion of TCGF reactivity, while the antigen independent proliferative phase is resistant to high concentrations of the same mAb. Furthermore, the reduced TCGF dependent proliferation,

that reflect CTL precursors which are resistant to anti-Lyt-2 mediated inhibition, is paralleled by a comparable decrease of cytolytic activity in the same culture. On a per cell basis, however, they have the same cytotoxic potential as those generated in parallel control cultures. Thus, it is highly unlikely, that binding of anti-Lyt-2.2 mAb's to CTL *per se* does provide a negative signal to these cells, since no inhibition of proliferation or maturation is observed, once initial activation has taken place. The latter finding also excludes that the antibody inhibits functional binding of growth or maturation factors. These results demonstrate, therefore, that anti-Lyt-2.2 mAbs do not simply reduce proliferation or cytotoxic activity, but that they prevent a large fraction of CTL precursors from being activated and initiate TCGF dependent clonal expansion. In addition, we could also confirm previous reports, in that precoating of effector CTL with anti-Lyt-2.2 mAbs, prior to the killer assay, were found to inhibit specific cytolysis, while precoating of Lyt-2$^+$ target cells had no effect. Since blocking of the Lyt-2 antigen inhibits both phases in CTL responses that require antigen recognition, namely, initial triggering and target cell cytolysis, the most reasonable interpretation for these findings is that the blocking effects of anti-Lyt-2.2 mAb are exclusively due to interference with the clonally distributed, antigen-specific receptors on these lymphocytes.

Several investigators have interpreted the observed inhibition of specific effector function by CTLs, as a strong indication that the Lyt-2 antigen constitutes a constant part of the antigen specific receptor. This, however, is highly unlikely because of the lack of "allelic exclusion" in both expression (154) and functional activity as target molecules for inhibition (W. Haas, personal communications). Furthermore, MacDonald *et al* have demonstrated that some CTL clones are resistant to anti-Lyt-2 mediated inhibition, thus indicating that the Lyt-2 antigen has no obligatory role in target cell cytolysis (159). I believe, therefore, that the most reasonable interpretation for our findings, is that cell surface structures on CTL precursors carrying Lyt-2 determinants are part of a functional membrane complex that contains the clonally distributed specific receptor, which delivers the

activating signals. In addition, it is possible that the Lyt-2 antigen in this complex, has a stabilizing function in the initial contact between the CTL precursor and the antigen carrying cell, thus increasing the probability for activation. If this would be the case, it follows that the stabilizing function of the Lyt-2 antigen is also operating at the level of effector-target cell interactions in the cytolytic phase of CTL activation. This interpretation is in agreement with the findings by MacDonald et al (159). These authors have shown, by using crossreactive Lyt-2^+ CTL clones, that some clones are resistant to inhibition by anti-Lyt-2 antibodies when lysing a given target, but can be readily inhibited by the same antibodies, if an antigenic crossreactive target cell is used. The authors propose (150) that different affinities for the respective target cell accounts for this variation and, therefore, that Lyt-2 antigens are only functionally relevant in effector-target cell interactions of lower avidity which require the "stabilizing" function of the putative complex.

7. Involvement of Lyt-2 antigens in lectin mediated CTL activation and target cell destruction

The widely spread use of polyclonally activating lectins for lymphocyte stimulation, makes it important to find similarities or differences in activating mechanisms between lectins and specific antigens. The selective inhibition of antigen recognition by an anti-Lyt-2.2 mAb, prompted us to perform a parallel study with three lectins, all potent inducers of T cells to TCGF reactivity. As discussed in the previous section, it is essential for this kind of approach, to use an experimental protocol that selectively induce Lyt-2^+ CTL. A suitable protocol is to expose an unselected T cell population to the activating lectin for 4 h, followed by extensive washing and subsequent recultivation in the presence of TCGF (VI). As argued above, we consider that the TCGF dependent response, obtained under these conditions, reflects exclusively the activity of CTL precursors.

In a striking analogy to antigen dependent induction to TCGF reactivity, anti-Lyt-2.2 antibodies exert a dose dependent inhibition on the initial lectin mediated triggering to TCGF reactivity. The induction to TCGF reactivity, by all three lectins, was reproducibly and profoundly inhibited by blocking the Lyt-2 antigen, while the TCGF dependent proliferation was totally resistant to inhibition by the same antibody. We were able to exclude inactivation of soluble lectin by the mAb, because solely CTLs expressing the relevant Lyt-2 allele are blocked. Furthermore, other antibodies of the same isotype, with specificity for proteins with abundant representation on CTL plasma membrane, have no effect in the same experimental system. As other nonspecific inhibitory effects are excluded by the same arguments used in the previous section, I find it reasonable to suggest that lectin dependent initial triggering is blocked at the same level as antigen dependent triggering, namely by interference with a receptor complex which includes clonally distributed specific receptors.

Hollander et al have recently reported that a ConA induced response is resistant to anti-Lyt-2 mAb (163,164). These results, however, are actually not in contradiction with ours for two reasons. Firstly, measurement of the overall ConA response, that is known to include other cellular subsets than Lyt-2^+ CTL, can not provide clearcut conclusions. Secondly, those experiments were performed by incubating the anti-Lyt-2 antibodies and the lectin with the cells, for the whole culture period. In the same type of experiment, we could only detect a marginal inhibition with anti-Lyt-2.2 mAb's. This result, however, is quite expected since soluble ConA has very high affinity for the specific sugar residues on cell membranes, and will likely successfully compete with any antibody, if present during a prolonged culture period. In order to avoid such problems, we have routinely preincubated the cells on ice, in the absence or presence of antibody for 30 min before addition of the lectin. Moreover, an extended preincubation period with lectins, reduces the blocking effect of anti-Lyt-2.2 mAb's, most likely by the same reason discussed above.

The similarities in anti-Lyt-2 inhibition of antigen or lectin-driven CTL responses was further substantiated by analysing the effect of monoclonal antibodies in ConA mediated cytolysis. As could be expected by the inhibition at the level of CTL precursors, also the cytolytic activity by mature CTLs on ConA coated target cells is significantly susceptible to anti-Lyt-2 inhibition. These results are at variance with some previous reports concluding that addition of ConA to killer assays "overcomes" anti-Lyt-2 mediated inhibition of specific cytolytic activity (160,165), but they are in agreement with others where this inhibition was still apparent in the presence of ConA (158,166). We have also observed with CTL generated in conventional MLC that ConA can "overcome" most of the anti-Lyt-2 mediated inhibition, in contrast to the highly active CTLs generated by TCGF expansion of polyclonally or MLCuv activated T cells. The significance of these discrepancies are, at present, not completely clear to me. I believe that the discrepancies outlined above might be due to different performances of lectin-mediated killer assays. We have noticed, similarly to the inhibition of induction of CTL precursors, that anti-Lyt-2 inhibition of ConA facilitated cytolysis is only obtained if the antibodies are allowed to bind to effector cells in the absence of lectin. Moreover, we use target cells precoated with ConA rather then agglutinating concentrations of lectin added simultaneously to mixtures of both effector and target cells, a procedure which is generally inhibitory for specific cytolysis (93,167,168).

8. Parallelism between antigen and lectin mediated T cell activation

Activation of T cells by lectins has often been regarded as a non-immunological reaction, due to nonspecific direct interactions of the lectin with the cell membrane which result in a mitotic signal. More recently, however, in analogy with antigen-specific T cell responses, Ia^+ accessory cells were found to be involved in the process of lectin induced activation (33,70-74). Finally, the demonstration (33) that

growth factors, rather than the lectins, are responsible for the mitotic activity has excluded the hypothesis that lectin binding per se would provide mitogenic signals to the responding cells. Although it is quite clear that lectin induced proliferation is a result of cellular cooperation similar to an antigen specific response, nothing is yet known about how lectins mediate their activating signals. On the other hand, it appears established that lectin binding to responding cells does activate them to acquisition of growth factor reactivity.

Two general hypothesis can be constructed to explain in terms of receptors the mechanism behind lectin induced T cell activation. Firstly, the lectin activates by triggering a putative "activation receptor", as proposed for LPS activation of B cells (169), or by nonspecific rearrangement of the cell membrane. In these cases, the immunological specificity would be bypassed, since the clonally distributed specific receptor does not need to "see" the inducing ligand or to be in any way involved in this process. One of the problems with this model is that a number of agglutinating, but nonmitogenic, lectins have been characterized (66). Together with the demonstration by Möller et al (170), that no correlation exists between binding and "mitogenic properties" of lectins, excludes that activation signals can be generated by extensive crosslinking of the plasma membrane. The differential requirements for ConA stimulation of CTL and TH cells (IV) and the Lyt-2 mediated inhibition of lectin activation of CTL precursors (VI), makes it very unlikely that "mitogenic" lectins deliver a triggering signal by direct interaction with a pan clonally expressed "activation site". The second hypothesis implies the participation of clonally distributed specific receptors, which are 1) either directly recognized by the lectin or 2) recognize the lectin, or lectin associated with cell surface structures, as a kind of universal antigen. The first and simplest alternative would assume that, of the many lectin binding sites present on CTL precursor membranes, one type would be the constant part of the clonally distributed specific receptors. The mere binding of the lectin to specific sugar residues on this constant region, abundantly represented on all T cell clones, should thus be enough to deliver a triggering

signal. The second alternative implies that the lectin, by interacting with MHC molecules on antigen presenting cells, creates new antigenic determinants that will be recognized by the responding T cell as "modified self" or allo-MHC determinants, in analogy with the postulates of Bevan *et al* (171) in antigenic systems. The polyclonallity of the response should consequently be a consequence of the very large number of all the possible spatial and sterical interactions of the lectin with MHC molecules, generating a high number of new antigenic determinants.

In view of my suggestion that Lyt-2 antigens are part of a molecular complex on CTL membranes that includes specific antigen receptors and mediates activation signals, the results obtained with lectins make a strong case for interaction of lectins with clonally distributed specific receptors. This idea is further supported by the fact that many lectins and antibodies bind to CTL membranes without functional consequences (66,155,172,173). Showing the requirement for specific interactions with specialized membrane structures in the induction process, however, we are not able to distinguish between the two alternatives outlined above, namely whether the lectins bind to the receptor or the receptor recognizes an antigenic complex created by the lectin.

If my conclusions and the general hypothesis are correct, the recognition requirements of CTL at the effector cell level imply, that activating lectins must also be competent to mediate the same effects as antigen at the level of target cell recognition and lysis. A number of observations made by others, some of them listed below, support this prediction and the idea that the inhibition of ConA mediated "nonspecific" cytolysis by anti-Lyt-2 antibodies is exerted at the level of clonally distributed specific receptors.

The orginal findings (174,175) that mitogenic lectins induce nonspecific target cytolysis were first explained by the agglutinating properties of the lectin. It became clear, however, that agglutinogenic but nonmitogenic lectins were unable to mediate nonspecific killing

(176,177). These findings, together with the demonstration by Kuppers and Henney (178,179), that cytolysis can only be achieved in the direction of antigen recognition, led to the conclusion that the effector cells need to be "reactivated" in order to express their lytic potential. In a recent report, Beretta et al (164) have observed that CTL, if precoated with ConA, completely fail to lyse the specific target cell. This phenomena has also been observed by others (168,180) and the most likely explanation is that ConA binds to and blocks structures, critically involved in recognition and lysis of target cells. In contrast, however, precoating of the target cells prior to the killer assay results in expression of cytolytic activity suggesting that effector CTL can functionally recognize lectin on target cells and be "induced" to cytolysis. If, as argued above cytolysis only operates in the direction of recognition, these observations support my suggestion that the specific capacity of mitogenic lectins to mediate "nonspecific" killing, is dependent on a functional binding by these lectins to clonally distributed specific receptors.

The experimental results in this thesis do not provide any answer to the question whether or not MHC molecules are involved in the process of lectin induced T cell activation. However, the observation by Kimura and Ersson is very provocative in this context (181) as it adds new arguments to the above hypothesis. These authors have demonstrated, that ConA-induced proliferative responses is inhibited by anti-H-2 antibodies, directed against either Class I or Class II antigens on responder cells. Furthermore, by using F1→ parent chimeras, they showed that ConA responses were only inhibited by anti-H-2 antibodies directed to the parental haplotype in which the responder cell had differentiated, while normal F1 spleen cells were inhibited by antibodies against either parental haplotype. This finding of ConA-induced "restricted" responses indicated that the inhibitory effect of the antibodies were not exerted by mere binding to the responding cells, but rather upon interference with some type of H-2-presenting function and their recognition by lectin-induced T cells. Similar studies performed at our laboratory have led to the same conclusion

(93, and E.-L. Larsson, to be published), which is best accommodated in the context of the general hypothesis precented here, namely that ConA-dependent activation of CTL involves clonally distributed, antigen-specific receptors.

Many questions still remain to be answered, and new possibilities are suggested by these arguments. Our observations, however, might have opened future perspectives, as systems employing polyclonally activating lectins may be after all of great value in the analysis of T cell repertoires and MHC restriction phenomena.

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