SUPPRESSOR T-CELLS AND THEIR FACTORS IN TUMOR-BEARING MICE

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A thesis submitted to the Faculty of Graduate Studies, University of Manitoba, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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To my parents

and my wife

TABLE OF CONTENTS Page

Abbreviations

ARC - antigen reactive cell

ATS - anti-thymocyte sera

B cell - Bursa equivalent cell

C' - complement

CI - cellular interaction

CMI - cell-mediated immunity

HTA - homocytotropic antibody

MLC - mixed leukocyte culture

MHC - major histocompatibility complex

PFC - plaque-forming cell

T cell - thymus derived cell

ABSTRACT

The ability of immune mice of the A/Jax ($H\text{-}2^a$) strain to reject the transplantable methylchloranthrene-induced fibrosarcoma, S1509a, was shown to be specifically and negatively influenced by the transfer to these animals of thymus cells from non-immune syngeneic mice bearing this fibrosarcoma. The characterization of this population of cells revealed that the active suppressor cells possessed the θ antigen, that they had a relatively low density, that they were cortisone-resistant and located primarily in the thymus, spleen, lymph nodes, and bone marrow of the tumor-bearing host. The mechanical disruption of these suppressor cells resulted in the release of an immunosuppressive factor (ISF) with identical properties to suppressor cells. The specificity of the factor was demonstrated by the ability of S1509a cells to deplete the cell extract of its activity by absorption, whereas other cultured tumor lines lacked this ability. In terms of its filtration behavior through Sephadex G-200, the molecular size of ISF was deduced to be in the range of 10^4 - 6.7 x 10^4 daltons. The interaction of ISF with a rabbit anti-mouse $F(ab')_2$ reverse immunosorbents did not result in its removal. However, ISF immunosuppressive activity was destroyed by digestion with pronase but not with RNase. Passage of ISF through a reverse immunosorbent prepared by insolubilization of antibodies directed at the products of the H-2K-I end, but not of the H-2D end resulted in a loss of suppressive activity. Hence, it was concluded that the factor was either a product of the H-2 complex, and in particular the K- or I-regions, or was closely linked to this complex on the cell surface. I^{125} cell surface radiolabeling allowed further

confirmation of the factor as a surface protein. The suppressor factor possibly represents an antigen specific receptor which may be important to the immune response to the S1509a tumor. The relevance and potential manipulation of this factor as it might apply to human neoplastic disease is also stressed.

In addition, the primary response to small tumor inocula has been studied. The effector mechanism responsible for elimination of small inocula can be blocked efficiently by solubilized tumor membrane, but is not modulated by adoptively transferred suppressor cells. Furthermore, only growing tumors which escape primary elimination induce suppressor cells.

Taken together, these data imply different regulatory mechanisms for the diverse effector mechanisms acting at various times in the immune response to tumor antigen.

I.

THE IMMUNE RESPONSE: T CELL - B CELL INTERACTIONS

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The nature of the complex process which results in the production of an immune response, after higher organisms encounter antigen, is not clear. The precise mechanisms by which antigen-specific activation of lymphocytes is achieved and by which such activation is regulated has not been delineated. A large body of evidence exists indicating that specific interactions of immunocompetent cells occur for the production of a maximal immune response. The mandatory cooperations for some antigens (*i.e.*, T-dependent antigens) between thymus derived (T) cells and bone marrow derived (B) cells (or, more correctly derived from Bursa equivalent organs) in the development of antibody responses has recently been intensely analyzed (4,8). The pioneering work of Good (1,2) and Miller (3) led to the recognition that the thymus plays an important role in the development and maintenance of the immunological system. Claman (4,5) clearly demonstrated that the antibody response to SRBC in a murine system was related to thymic influence.

The active T cells are ontogenically the mature cell in the thymic medulla and apparently represent precursors of the cooperating cell population. It is now known that T cells [as judged by the θ marker, recognized by an alloantiserum specific for T cells (6,7)], also exist in areas other than the thymus. For example, thoracic duct cells, spleen, lymph nodes and peripheral blood do contain thymus-derived cells (7,8). It is also quite clear that neonatal thymectomy leads to a profound decrease in θ antigen bearing

reactive T cells in the spleen (10). B cells come to different areas in lymphoid organs and are not θ antigen positive (9-11). B cells represent a portion of the lymphoid population destined to produce antibody.

In general, an antigen may be considered to consist of two functionally different parts, *i.e.*, one part, the carrier, bestowing the activating capacity to T cells, and a smaller portion, the hapten, which is recognized by immunoglobulin receptors on B cells. *In vitro* analysis of the immune response led to the conclusion that glass adherent cells, consisting primarily of phagocytic cells, as well as nonadherent cells are necessary to cooperate in the generation of an immune response. Thus, the work of Mosier and others (12,13) showed clearly that adherent cells were necessary for the generation of plaque-forming cells (PFC). It is known from the work of Unanue (14,15) that antigens which are extensively phagocytosed in the reticulo-endothelial system (RES) are apparently 'better' antigens and it has been shown that at least some amount of the originally administered antigen remains on the surface of such cells. Therefore, it would seem that for some antigens, a complex event occurs in which the T cell, B cell and macrophage interact to recognize the separate functional units of the relevant antigen. Pertinent to this problem is the observation by Miller *et al.* (16) and Shearer *et al.* (17) that the antigen reactive T cell (ARC) must differentiate and undergo proliferation in order to maximize the informational transfer. These observations lead to the consideration of the nature of the hapten-carrier cellular interactions. Mitchison (18-20)

hypothesized a cooperative event with two cell types recognizing different antigenic moieties. Mitchison utilized an experimental design in which one group of mice was immunized with a hapten-carrier (H_1-C_1) conjugate and a second group of mice immunized with a different carrier (C_2). The population of cells immunized with C_2 were, therefore, distinct from the population of cells immunized with the hapten H_1-C_1. The two spleen cell populations isolated from the first and second group were transferred simultaneously into irradiated recipients, boosted one day later with a conjugate of the hapten and the second carrier (H_1-C_2) and then bled after another 8-10 days. A specific antihapten response was demonstrated by the challenge of the recipeints in this manner. Moreover, it was observed that the maximal stimulation for a specific antibody response required a physical union of the two determinants involved in cooperation. Therefore, it appeared that one lymphocyte population "recognized" the carrier and another population "recognized" the hapten. Thus, the collaboration of these two types of cells was explained by postulating that T lymphocytes bind an antigenic molecule through their carrier-specific receptors and concentrate or "focus" the antigen on their surface so that the haptenic determinants can be bound by the hapten-specific receptors of the B lymphocytes.[22] Subsequently, the population of carrier-reactive cells was shown to be thymus derived by Raff (5) who demonstrated their susceptibility to anti-θ serum and C.

However, this antigen-focusing theory is not totally acceptable since binding of antigen by T cells is relatively difficult to demonstrate (23) and the concept that the role of the T lymphocyte is simply to concentrate antigen on its surface is probably a great oversimplification. Nevertheless, recent work by Ungar-Waron *et al.* (24) tends to support the cooperation hypothesis. These authors demonstrated that the insertion of a rigid polyproline spacer between the hapten and carrier caused no discernible change in the carrier-induced effect. This confirms the observation that T cells recognize with specific moieties of the carrier portion of the antigen. For some time it had been argued that the environment immediately adjacent to the hapten may be recognized in T-B collaboration (25). The local environment theory has been recently reconsidered by Janeway (26) who stated that, while the specificity of T cells bears a superficial resemblance to hapten-specific B cells in that these T cells can respond to hapten on a variety of unrelated carriers, the precise specificity of such T cells can be shown to involve the hapten plus contiguous regions of that carrier. This almost certainly includes the amino acid residues by which the hapten is joined to the carrier.

Subsequent experimentation has focused on the interaction between T and B lymphocytes, both with regard to its mechanism and its relevance to the immune response. The observation by Katz *et al.* (27) of the allogeneic effect has revealed other aspects of T-B cooperation. The phenomenon termed the allogeneic effect

was the demonstrated increase in antihapten responses which followed the passive transfer of immunocompetent allogeneic lymphoid cells into antigen-primed animals. These observations demonstrated that the most critical event in the allogeneic effect is the interaction between foreign T lymphocytes or their products, and the primed B lymphocyte of the host. In particular, the allogeneic effect has been shown to provide the helper function for antigen-specific B cells. This effect is thought to be mediated by a T cell product (28,30).

Although the allogeneic effect can enhance antibody responses, it can also exert a suppressive influence (29). Therefore, these observations led to the speculation of the regulatory role of T cells in both enhancing and suppressing (25,27,28) the B cell response to antigen.

The nature of the apparent cooperation of B and T cells may only be the result of factors produced by the T cell which then stimulate the B cell (28). Recently, Benacerraf, Katz and others have also considered that certain H-2 I region gene products, or moieties closely related to them, may represent the interaction sites involved in T-B cell interactions. According to their hypothesis the Ir gene products or associated I region gene product(s) from the T cell may govern the interaction with the B cell at the histocompatibility 'acceptor' site on the B cell surface in the secondary response to antigen (28,33). A comprehensive cellular scheme of T-B cooperation, based on experimental observations, has been described by Katz and Unanue (31). Antigen is concentrated

by macrophages and other accessory type cells as previously suggested by Nossal (in 4); and, therefore, antigen committed T and B lymphocytes of the recirculating pool are trapped along the sites of antigen concentrations. T lymphocytes, triggered by this cell-bound antigen, then become activated and the activated T cell and/or its mediators regulate the triggering of nearby B lymphocytes at some time during or after the interaction of these B lymphocytes with antigen. These events are modified in an immune animal by the presence of antibodies that favor the concentration of antigen on macrophages, or possibly by a large number of existing primed B cells which then serve as antigen concentrating cells and by primed T lymphocytes which have varying threshold levels of activation.

This model is in some disagreement with the Feldmann model (32-34) in which a putative T cell receptor IgT (35,36), after interaction with the antigen, is shed from the T lymphocyte surface and, thereafter, bound by a third party cell such as a macrophage or a dendritic cell. Binding of the B cell receptors with the antigen would occur at the surface of the accessory cell; which has the IgT-Ag complex arranged in a lattice on the cell surface (36). A problem not solved by the Feldmann model is that normal physiologic T-B interaction is relatively difficult to demonstrate across major histocompatibility barriers, particularly where different K region haplotypes are involved (31). If the difficulty across the histocompatibility barrier is considered in terms of Feldmann's IgT hypothesis, then the block must relate to the interaction between the IgT-antigen bearing macrophages and the responding allogeneic

B cell. Although it is evident from the work of Shevach *et al.* (38) that there are genetic constraints of macrophage-T lymphocyte interaction, this would suggest that the effect in physiologic cooperation in allogeneic systems may relate to the actual interactions between T and B cells; a possibility that cannot be fully explained by the IgT-antigen complex theory (34).

Recently, another comprehensive and elegant biological scheme has been proposed by Bretscher and Cohn (39), whereby a B cell receives two distinct signals. The first signal is the interaction of the B cell with antigen and the second signal is directed by T cells, alone or via the macrophage. The precise genetic restrictions for effective secondary T-B cooperation (33) (see page 33) clearly are different than the restrictions imposed on primary T-B interactions (40) in which good cooperative T-B cell interactions have been obtained across major histocompatibility differences. Perhaps the most noteworthy recent observations on the subject of T-B cooperative interactions has dealt with the possibility that restrictions in collaboration depend upon the antigen alteration of self components which occur in priming. The context in which a T cell sees the antigen is related to the association of that antigen with MHC determinants so as to result in the recognition of altered-self. The secondary responses are optimized when such primed animals are rechallenged with similar antigen altered MHC determinants. Therefore, if spleen cells from mice of haplotype A are primed with macrophage bound antigen of haplotype B origin, they will be restricted in

their capacity to respond and develop antibody responses to that antigen, unless challenged with only antigen-bearing macrophages of strain B (33,40).

Therefore, it is conceivable that the antigen is 'seen' in many cases of successful antibody responses as a modified component of self.

II.

T-T CELL INTERACTIONS

T-T Cell Interactions

The expression of T cell responses is manifold. Included in these responses are the delayed type hypersensitivity (DTH) response, the graft-versus-host (GVH) response and T cell-mediated cytotoxicity (CMC).

It is known that in the GVH reaction, T cells of two types interact to mediate this cell-mediated response. In one model, studied by Cantor and Asofsky (177), two cell populations, termed T1 and T2 were described. The T1 population recirculates slowly and represents the precursors of the effector (*i.e.*, killer) cells which inflict immunologic injury, whereas the T2 cells provide the amplification effect and are present in a rapidly recirculating T lymphocyte pool; T2 are present in excess in peripheral blood and are more sensitive to ATS than killer cells, an observation that will be shown to be important in this thesis.

Synergy, during the *in vitro* cytotoxic allograft responses, has been shown by Wagner (179) to be a result of the cell interaction between thymocytes and peripheral T cells. The cytotoxic activity was mediated by effector lymphocytes (CL) derived from peripheral T cells, but required the presence of immune reactive thymocytes in order to be optimally immunized *in vitro*. Wagner suggested that thymus cells may act also as "amplifier" cells. The recent description that peripheral T cells are composed of functionally distinct subclasses, each defined by a characteristic cell surface pattern of Ly antigens, has allowed further definition

of the T1-T2 population

Cantor and Boyse (178) have shown that cytotoxic T lymphocytes generated in allogeneic MLR cultures belong to the Ly $1^-2^+3^+$ subclass, whereas most of the T cells responsive to I-region incompatible stimulators express the Ly $1^+2^-3^-$ phenotype.

Gelfand and Steinberg (180) have shown that old NZB/W mice reject allogeneic skin grafts more slowly than young NZB/W mice and suggested that this effect was due to a deficiency in one cell type which participates in allograft rejection; this hypothesis was supported by the demonstration that prompt graft rejection was restored by the injection of small numbers of spleen cells from young NZB/W mice into old NZB/W mice.

Dennert and Lennox (181) demonstrated that by immunizing Balb/c mice with low doses of irradiated or formaldehyde-treated P815 cells, helper activity was generated as measured in an *in vitro* assay to a hapten coupled to the P815, but no killer cell activity could be demonstrated. Moreover, if the mice were immunized with P815 tumor alone, the spleen cells showed cytotoxicity by no helper cell ability. The authors concluded that these two populations, helper cell and killer cells, interacted operationally, yet were apparently mediated by independent subpopulations of T cells. Furthermore, it has become apparent that helper cells express the Ly 1 phenotype, whereas cells of the Ly 23 class express cytotoxic activity, establishing that they truly represent separate subsets of T cells (302).

Relevant to this discussion of T cell-T cell interaction is the observation by Stobo *et al.* (182). These authors demonstrated that

splenic T cells, which respond to allogeneic cells in a mixed lymphocyte (MLC) reaction, and splenic T cells from alloimmunized animals which specifically lyse allogeneic target cells, are different T subpopulations. The cells responding in MLC are contained within the recirculating radiation sensitive T cell population, whereas the cytolytic cells reside within the sessile, relatively radiation-resistant population of T lymphocytes. It was found that the cells reactive in MLC reactions are required for the generation of maximal cytolytic activity.

In another study utilizing an *in vitro* autosensitization model, Cohen *et al.* (183) have shown that fibroblasts could be used to sensitize spleen or thymus cells. These spleen or thymus cells, acting as sensitized lymphocytes were injected into the foot pads of syngeneic recipients. Specific effector lymphocytes appeared to be recruited by these sensitized lymphocytes from amongst lymphocytes present in the regional lymph node. These workers concluded that there were similar numbers of antigen-reactive cells in the thymus and the spleen and that the antigen reactive cells did not differentiate into effector lymphocytes. Proliferation of these recruited lymphocytes appears to be needed for the generation of an effector response. These studies, as well as those of Cantor and Boyse (178), imply that separate subsets of T cells synergistically interact to produce cytotoxic responses. It has been proposed that helper cells recognize I-region H-2 differences and that killer cells respond to the K or D region antigens in such interactions. Although mechanisms by which cell interactions and cell functions are regulated

is not clear, it is evident that different cell subpopulations participate in the generation of the immune response. An attempt will be made to discuss the regulation of the immune response in this context.

III.

REGULATION OF THE IMMUNE RESPONSE

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Regulation by Antibody

The first clear statement that specific antibody (Ab) could have a regulatory effect on antibody formation during a conventional antibody response to a metabolizable antigen, is attributable to Graf and Uhr (114,120). By employing immunoadsorbents, they showed that removal of specific antibody from an animal prompted further production of that specific antibody. Previously, many authors had arrived at the conclusion that 7S antibodies (IgG_2) could exert a suppressive effect (115-118) on antibody production in mice. This, however, was complicated by the observation that 19S antibody had a varied effect, *i.e.*, stimulatory to the synthesis of certain types of antibody and inhibitory to the synthesis of other types of antibody (116). Moreover, some authors felt that 19S Ab synthesis itself was less resistant to the effects of suppression by antisera than 7S synthesis, a phenomenon possibly related to the greater affinity of IgG antibody as compared to IgM antibody. Britton and Möller (117) showed that following a single injection of a bacterial polysaccharide antigen into CBA mice, cyclical fluctuations of humoral 19S antibody synthesis was evoked. Their interpretation was that this phenomenon represented a feedback suppression of active antibody synthesis by endogenously-produced 19S antibody and postulated that during various phases of the immune response, the biologically stable antigen (E. Coli endotoxin) was "covered" by the 19S antibodies directed against it, that

subsequently the antibodies masking the antigen were catabolized faster than the antigen. Thus, the antigen would become free and would stimulate immunologically competent antibody-forming cells. The effectiveness of passively transferred antisera demonstrated a profound suppressive effect of the 7S component on active antibody formation, and this effect was presumed to be attributable to the IgG_2 fraction.

The role of the different portions of the antibody molecule in its effects on the regulation of antibody formation was assessed by Sinclair (119),in a pioneering study, who demonstrated that in the murine system, the whole 7S antibody molecule was 100 times more suppressive than the corresponding $F(ab')_2$ fragment obtained by pepsin digestion. This evidence suggested that the phenomenon of antibody-mediated suppression was not the result of only the masking of the antigenic determinants and that the Fc portion was fundamentally involved in suppression of the antibody response. Although not substantiated by experimental data, Sinclair postulated that the Fc portion may prevent the catabolism of antigen, or may suppress Ag-sensitive cells from reacting to antigenic determinants.

Another theory suggests that passively administered antibody results in reduction of the absolute number of B-immunocompetent cells capable of responding to a given antigen; this mechanism at the cellular level is considered to be a "central" mode for induction of specific nonreactivity. Diener and Feldmann (121-123),in a series of elegant *in vitro* experiments, investigated the cellular mechanism

of antibody-mediated suppression. They then extended the *in vitro* techniques by transferring cultured cells into lethally irradiated recipients and testing their reactivity *in vivo*. Using POL (polymerized flagellin of Salmonella adelaide) they demonstrated that anti-POL antisera induced suppression of the anti-POL response both *in vitro* and *in vivo*. The kinetics of such immune suppression *in vitro* followed an exponential time course. It was shown that these complexes were effective when formed in antigen excess, suggesting that anti-POL could be immunosuppressive *in vitro*, without being in a concentration sufficient to cover all the antigenic determinants. These authors postulated that the specific suppression was due to the formation of a rigid lattice of membrane-bound immunoglobulins through interaction with the homologous antibody-antigen complexes. With the relevant cells, they also found evidence for depletion of specific B-antigen reactive cells.

The importance of this suggestion, that antisera might have an effect on cellular receptors, has been stressed by others. Pierce, Solliday and Asofsky (124) demonstrated that goat anti-mouse μ-chain serum could suppress primary PFC responses of the IgM, IgG_1 and IgA classes in mouse spleen cultures. This was not due to a cytotoxic effect of the sera as judged by the Cr^{51} cytotoxicity test. The suppressive effect of this anti-μ serum was thought to occur at the level of the precursor B-lymphocyte. The conclusion of the study was that anti-μ combined with the μ-chain determinants of the IgM receptors for the antigen. Thus, it would appear that saturation or interference with the function of the receptors of B cells would

prevent stimulation of these cells by antigen and, thereby, result in a suppression of the subsequent antibody production.

As regards the possible effect of antisera on CMI responses, some authors have suggested that passive antisera may play, also, a role in CMI regulatory functions other than graft rejection (131). The present status of the role of antibody in CMI responses remains ambiguous, particularly if one examines syngeneic donor-recipient systems, where varied and inconsistent effects are seen (125-133). In the alloantigen-injected rat transplantation model, the possibility of an anti-idiotype-like antibody developing, and subsequently exerting a regulatory effect, has also been proposed and remains an exciting immunotherapeutic possibility (134). This particular concept has been extended by Andersson *et al.* (130) who showed that mice injected with their "own" lymphoblasts, which carry receptors with antigen binding specificity and idiotype positive markers, produce auto-anti-idiotypic antibodies. This has allowed the experimenters to induce specific unresponsiveness to transplantation antigens. Potentially this may be of enormous value in future treatment of autoimmune disease , allergic disorders or in transplantation therapy.

While investigating the role of various antisera in the regulation of anti-hapten homocytotropic antibody (HTA) to dinitrophenylated Ascaris serum extracts (DNP-As), Tada and co-workers (135,136) discovered that homologous antibody against the same

antigen exerted a suppressive effect. It was also found that animals given anti-DNP-As antibody simultaneously with the initial immunization of DNP-As produced the lowest quantitative response. However, using antigen-antibody precipitates in which hapten, or carrier, or both were covered by an excess of antibodies directed at the respective determinants, it was found that if given at day 0, anti-carrier antibodies could be suppressive, whereas anti-hapten antibodies did not suppress, but rather enhanced the succeeding HTA formation. In a second protocol, the determinants were covered at the time of a second injection and only anti-hapten antibody was suppressive.

These results indicate that the carrier determinants are more important in the initial antigenic stimulation necessary to generate an effective anti-hapten response. Vaugnat *et al.* (137) employed a guinea pig model which involved the use of passively administered immune complexes containing IgG_2 or IgG_1 anti-DNP antibodies, to study the effect on anti-DNP antibody production after DNP-BGG challenge. They demonstrated that both guinea pig $F(ab')_2$ and IgG_2 anti-hapten antibodies depressed both the IgG_1 and IgG_2 anti-hapten responses, whereas IgG_1 anti-hapten antibodies had a differential effect on these two classes, *i.e.*, the active IgG_2 anti-hapten response was suppressed and the IgG_1 response was delayed but its level was enhanced. Hence it is clear that antibodies belonging to different immunoglobulin classes have different effects on the antibody response.

Vaugnat *et al.* (138), in another series of experiments, used DNP-BGG in guinea pigs and studied the anti-carrier effect on the antibody response to hapten. In this series, a sustained suppressive effect of passive IgG_1 anti-carrier antibodies on the active IgG_1 anti-hapten response was observed. The antibody would influence antigen localization through its class, and then at least for IgG_1 antibodies, influence the immune system according to its specificity, either anti-carrier or anti-hapten. It should be noted that the production of IgG_1 antibodies in the mouse is thymus dependent (139). It is conceivable that IgG_1 anti-carrier antibodies would shut off carrier-specific T cells, thus resulting in a depressed anti-hapten antibody response. As mentioned above, the differential effects of IgG_1 and IgG_2 sera suggest a regulatory role for the Fc fragment (119). Pincus *et al.* 140-142) have demonstrated that administration of a relatively small dose of antiserum to BSA into mice receiving an injection of DNP-BSA resulted in a concomitant enhancement of both the anti-BSA and anti-DNP antibody responses. It thus appears that under suitable conditions, the "helper effect" may be enriched by antisera to the carrier.

Haughton and Makela (148) reported recently that administration of anti-carrier or anti-hapten antibodies into mice resulted in different effects. Thus, high doses of anti-carrier antibodies caused a modest degree of immunosuppression. Similarly, high doses of anti-hapten sera were strongly immunosuppressive, but low doses augmented the response. According to these authors, suppression

and augmentation are due to distinct properties of antibodies, suppression being a function of anti-hapten antibodies and augmentation a function of anti-hapten antibodies directed against the specificities introduced into any of the protein carriers as a result of conjugation with haptens.

In a series of papers, Kappler, Hoffmann and Dutton (149-151), using a murine model and TNP-SRBC as the antigen, demonstrated that antibody (to the antigen) did not inhibit the priming of T cells for their helper effect in the response to TNP-HRBC. They proposed that antigen and antibody form complexes and that these complexes had affinities not only for antigen-specific receptors, but also for Fc receptors on B lymphocytes. The overall frequency of antigen-specific lymphocytes is exceeded by the great number of Fc receptors present on B cells. Consequently, complexed antigen is spread over the surface of a large number of B cells, via the Fc receptors, interfering with the precise presentation of antigen to antigen-specific receptors. However, as the authors have shown, T cell priming could still occur as it is less sensitive to antibody. This mechanism has certain similarities to that proposed by Dennert (152), whereby, using Cr^{51}-labeled SRBC in a murine system, he demonstrated an apparent IgM antibody-induced antigen-concentrating mechanism in the spleen resulting in an enhanced immune response. Evidently, IgG_1 does not concentrate antigen and possibly competes with the respective antigen receptor sites.

Recently, a study by Hamaoka *et al.* (153), has suggested that the suppression of anti-hapten antibody response by anti-carrier antibody seemed to be due to the disturbance of the cooperation between primed cells and hapten-primed cells, *i.e.*, antibodies attached to the carrier may interfere with the interaction between the carrier and T cells, abolishing this cooperation with B cells.

T Cell Dependency of Antibody Suppression

A model which deserves mention is that described by Schierman and McBride (143) for the chicken. In chickens, isoantigens determined by the B blood group locus behave as carriers for isoantigens determined by the A blood group locus. This occurs only if the antigens are present on the same erythrocyte and the recipients are not rendered immunologically tolerant to the B antigens (144). These authors showed that injection into G-B_1 chickens of red blood cells possessing two serologically distinct blood group antigens (A_2 and B_2), coated *in vitro* with anti-B ab leads to the suppression of the anti-B response and enhancement of the response to the A isoantigen. Recently, the same authors (45) have shown that the ability of passive antibody to enhance the production of antibody to non-coated determinants, on the same immunogen, depends on the presence of T cells. The amplification of the activity of B cells engaged in anti-A antibody synthesis is somehow mediated by the interaction of T cells with the complex of B blood group antigens with anti-B antibody.

Experiments which demonstrated thymic dependency of immune response suppression were reported by Gershon and Kondo (135) while analyzing antigenic competition. These authors have shown that antigenic competition between sheep red blood cells and horse red blood cells was thymic dependent. Moreover, they clearly demonstrated (136) that a certain dose of passive antibody allows antigen to interact with T cells, but prevents these cells from cooperating with precursor B cells in the formation of antibody. This explanation is in accord with the mechanism for antibody formation discussed earlier, which requires that antigens interact with both T and B cells.

Regulation by T Cells

As discussed previously, T cells or their soluble factors cooperate synergistically with B cells in the induction of antibody formation against a variety of antigens. Moreover, it has also been shown that there exists cooperation of different subsets of T cells in the generation of CMI responses. However, as already alluded to, some T cells may have suppressive effects on both the humoral and cellular manifestations of the immune response, *i.e.*, on B and effector cells; these effects will be the topic of the next section.

Suppressor T cells in T-B Responses

The work of Gershon and Kondo (146,147) first heralded the possibility of a suppressive influence of thymus-derived cells. In investigating these suppressive effects, Gershon and Kondo used thymectomized and lethally irradiated mice, which were protected

with syngeneic bone marrow grafts and immunized with SRBC. Administration of SRBC in the absence of T cells had no significant effect on the ability of bone marrow cells to reconstitute the mercaptoethanol-resistant fraction of the anti-SRBC response. However, if the mice had been reconstituted with a small number of thymocytes at the time of bone marrow reconstitution, they showed marked impairment of their ability to produce anti-SRBC antibodies. This suggested that some forms of tolerance required thymic lymphocytes.

In another series of experiments, the same authors (154-155) showed that the adoptive transfer of spleen cells from mice, rendered tolerant to SRBC, specifically prevented cooperation of normal thymocytes and normal bone marrow cells to mount an anti-SRBC response. It should be noted that thymus cells had to be present during the course of tolerance induction for the effect to be seen; the authors used the term infectious tolerance to describe this phenomenon.

It soon became clear that removal of thymic influence could heighten the ability to produce an antibody response in mice to certain thymic independent antigens. Thus, Baker *et al.* (156) used a single dose of anti-lymphocyte serum (ALS) at the time of immunization with pneumococcal polysaccharide type (SSS-III) and showed a marked increase in the anti-SSS-III specific response, as judged by an increase in the magnitude of the PFC response to an injection of SSS-III. It was proposed by these authors that the ALS inactivated a thymus-derived cell which normally acts to suppress the antibody

response produced following immunization with SSS-III. Furthermore, they showed that administration of 10^6 to 10^7 thymus cells resulted in the reduction of the PFC response by 20-70%, whereas equivalent numbers of peripheral blood cells produced a 40-120% increase. Hence, they concluded that there were two functionally distinct types of T cells, *i.e.*, suppressor and amplifier T cells.

In subsequent experiments, Baker *et al.* (157) characterized the nature of the T cell involved in the regulation of the response of mice to SSS-III. Athymic nu/nu mice did not show a significant increase on PFC upon ALS treatment. Moreover, the mode of action of thymic-derived suppressor cells was examined. Using Velban (vinblastine sulfate), a mitotic inhibitor, and ALS, Baker *et al.* (158) made other observations. By noting that ALS induced a continued proliferation of antibody-forming cells, a mitotic inhibitor was given 4 days after immunization and appeared to arrest completely the further development of PFC in ALS-treated mice. Therefore, the authors felt that suppressor cells might influence the extent to which antibody-forming bone marrow-derived cells proliferate following immunization. Very recently, the authors (158) have concluded that specific, low dose tolerance to SSS-III is a T cell-dependent phenomenon due to the action of suppressor T cells, and suggested that these cells might exert an influence on amplifier cells. Evidence which does not support this conclusion as one which applies to all T-independent antigens is found in the experiments of Barth *et al.* (160), who demonstrated that ALS could not

induce enhancement of the antibody response to E. Coli lipopolysaccharide, which is considered to be a thymus-independent antigen.

Using a different approach, Rich and Pierce (161) demonstrated that concanavalin A (Con A)-activated lymphoid cells could suppress or enhance the primary humoral immune responses of mouse spleen cells to heterologous erythrocytes in an *in vitro* model. Thus, while addition of mitogenic doses of Con A to spleen cell cultures at initiation resulted in suppression of PFC responses, addition of submitogenic doses of Con A led to enhancement of PFC generation to heterologous SRBC. However, addition of mitogenic doses of Con A after 48 hours led to marked enhancement of PFC response. In a later communication (162), the authors showed that the suppressive effect of Con A was due to the generation of thymus-derived lymphocytes since treatment of the cells with anti-θ serum and complement resulted in abrogation of the suppression. Moreover, they showed that suppressor T cells were resistant to irradiation (2000 rads) and that these cells were present in large numbers in peripheral lymphoid tissues, being most apparent in the spleen and almost absent in the thymus. More recently, Peavy and Pierce (163) have investigated another intriguing aspect of Con A-activated spleen cells. Using a one-way mixed leukocyte culture (MLC), these researchers have shown that the generation of cytotoxic lymphocytes *in vitro* can be influenced by Con A. Spleen cells from C57bl/6 ($H\text{-}2^b$) were incubated for 5 days with mitomycin C-treated DBA/2 ($H\text{-}2^d$) spleen cells and the resulting cytotoxic lymphocyte response

(CLR) was measured by lysis of Cr^{51}-labeled P815 ($H\text{-}2^d$) mastocytoma cells. With mitogenic concentrations of Con A,(1-5 μg/ml), added at initiation, maximal suppression of the development of cytotoxic lymphocytes was observed. Furthermore, 2.5×10^6 Con A-activated C57Bl/6 spleen cells added at initiation of the culture, showed a maximal suppressive effect. Peavy interpreted these findings as indicating that the suppressor cells had an abrogative effect on CLR.

In recent experiments, Ha and Waksman (164) have also demonstrated the participation of suppressor cells. Lewis rats treated with 100 mg bovine γ-globulin,intraperitoneally, were thymectomized and splenectomized. The thymus cells and spleen cells were transferred intravenously into a single untreated syngeneic recipient, which was challenged 24 hours later with antigen in complete Freund's adjuvant. Subsequently, the recipients were skin tested and bled. The recipients showed inhibition of specific Arthus and delayed hypersensitivity responses, as well as a delay in the formation of hemagglutinating and hemolytic antibody to BGG. Furthermore, Ha and Waksman (165) showed that suppressor cells were of low density and were relatively steroid resistant. These suppressor cells may demonstrate adherent-like qualities as well (268).

A phenomenon which also appears to involve the regulatory effect of suppressor cells is the abrogation of the synthesis of the Ig-1b allotype present on IgG_{2a} globulins. Using the observation that suppression of the Ig-1b allotype production in Ig^a/Ig^b hybrids derived from the mating of SJL (Ig^b) males with BALB/c (Ig^a)

females immunized to Ig-1b, a long-lived and presumably active process, Jacobson *et al.* (166-168) investigated the possible role of spleen "suppressor" cells. In these studies, spleen cells of chronically suppressed donors were mixed with cells of normal syngeneic donors and were transferred into irradiated BALB/c mice, in which production of the allotype could be followed. This transfer of suppressed and normal cells resulted in a transitory burst of Ig-1b synthesis after which the serum level fell. Herzenberg (168) then showed that the suppressor activity of spleen cells, from suppressed animals, was destroyed by incubation of the cell suspension with antibody to the Thy-1 antigen in the presence of guinea pig complement. This system, therefore, demonstrates a unique phenomenon, *i.e.*, that a T cell or its product can specifically suppress one of the possible two allotypes on immunoglobulins in heterozygotes. Moreover, Okumura *et al.* (197) has obtained evidence that these suppressor cells affect negatively the T helper cell rather than the B cell. The suppressor cell operates through factors coded by the I-J subregion of the H-2 complex (176,307).

Using spleen cells harvested at various intervals after immunization with BGG, Gershon *et al.* (169) assessed their response to phytohemagglutinin (PHA) *in vitro* in the presence or absence of the immunizing antigen. The PHA response of these spleen cells was suppressed if BGG was also placed in the culture. This result suggests that continued presence of antigen is mandatory for the continued generation of suppressor cells or maintenance of suppressor

cell activity. It is to be noted that in the absence of added antigen to the culture medium, the spleen cells showed an increase reactivity to PHA, which was maximal at 24 hours.

In a more recent communication, Gershon *et al.* (170) utilized a graft-versus-host (GVH) system demonstrating that parental thymocytes inoculated into F_1 mice, which had been lethally irradiated, reacted to the antigen contributed by the other parent in the F_1 hybrid. However, addition of F_1 thymocytes to the inoculum suppressed the response of a highly reactive cell inoculum and boosted the response of an inoculum responding less well. Hence, these authors concluded that T cells may emit bidirectional signals.

Perhaps the most extensive and in-depth analysis of T cell regulatory mechanisms may be attributed to the studies of Tada *et al.* (171-175) on the formation of homocytotropic antibodies in Lewis rats (172). These workers established (173) that splenectomy or thymectomy could enhance the anti-hapten homocytotropic antibody (HTA) response on immunization with DNP-As, if the operation was performed before or soon after immunization and they showed that thymocytes of rats hyperimmunized by repeated injections of DNP-As suppressed the anti-hapten HTA of syngeneic recipients of these T cells. From these results, these investigators concluded that a carrier-specific suppressor cell was responsible for the regulation of the hapten-specific HTA response.

In the course of study (173), it was also found that high doses of, and hyperimmunization with, carrier molecules, similarly

suppressed hapten-specific HTA formation against the hapten-carrier conjugate, while the same treatment with hapten coupled to irrelevant carrier did not. This carrier dependence of high dose tolerance had been induced in carrier-specific helper cells, but not in cells destined to become antibody-forming cells.

In another experiment (174) which was designed to study whether regulator cells in hyperimmunized animals could act as helper cells in tolerant animals, in which only the helper cell population had been impaired; it was found that passive transfer of thymocytes from animals hyperimmunized with the carrier (As) could reconstitute the ability of tolerant animals to make HTA against DNP-As, while thymocytes immunized with hapten on a different carrier (DNP-BSA) could not. Thymocytes from normal animals also reconstituted the immune response. It might be concluded, therefore, that helper and suppressor cells are identical, or alternatively the suppressor cell might represent a less mature form of the helper cell. If this is so, further differentiation might occur with maturation of suppressor cells into helper cells.

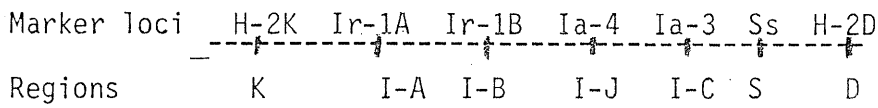
More recently, Okumura *et al.* (221) showed that a subcellular fraction of the extract obtained by mechanical disruption of thymocytes or spleen cells of Lewis rats immunized with Dnp-As also specifically inhibit the ongoing HTA synthesis against the same hapten carrier in Lewis rats hyperimmunized with DNP-As. The suppressive factor in this extract was not a conventional immunoglobulin, since it was not removed by reverse immunosorbents prepared by insolubilization of antibodies to rat IgM or to the Fab fragment of rat IgG.

In addition to being derived from T cells, it was shown to be carrier-specific. Because of the rapidity of action, the authors concluded that suppressive factor might act directly on B cells inhibiting their antibody synthesis. Tada has recently found that BALB/c ($H\text{-}2^d$) mice primed with KLH, generated suppressor T cells, which when passively transferred to syngeneic recipients could suppress the IgM or IgG anti-DNP response if such mice were immunized with DNP-KLH. Such suppressor cells carry or produce a subcellular component with similar biological activity. Immunochemical characterization of this factor has demonstrated the presence of H-2 I determinants—strict carrier specificity; and furthermore, that it may originate from the I-J subregion of the MHC (176,307,308).

Recently, Zembala and Asherson (301) have described that a subpopulation of T cells may be responsible for the depression of contact sensitivity to picryl chloride in mice. Thus, T cells from lymph nodes of mice rendered tolerant to picryl chloride, by the use of picryl sulfonic acid, could suppress the contact sensitivity response to picryl chloride in syngeneic recipients. Therefore, these authors concluded that suppressor T cells could modulate other T cell responses, including delayed type hypersensitivity. The view that the immune response may be regulated by antibody in some cases and by thymus-derived cells or their products in other cases, has obvious implications in relation to the present study which is directed toward the elucidation of the regulatory mechanisms which determine the immune response of the tumor-bearing host to the malignant cells.

The H-2 System

Görer (271) first described the H-2 system serologically and furthermore showed its importance in transplantation. The H-2 major histocompatibility locus is located on Chromosome 17 of the ninth linkage group. By the use of recombinant inbred strains, a number of genetic regions have been defined which are distinct inasmuch as functions or products unique to the regions can be separated by recombination. The loci for these regions and the regions below are:



Most of the traits ascribed to the K or D regions are probably under the control of single H-2K and H-2D genes, the expression of which are definitely cell membrane proteins. Each H-2 haplotype has multiple H-2 specificities, some specific for the haplotype (private specificities) and others shared with other haplotypes (public specificities). The idea of a common ancestral gene produced by mutational divergence and duplication--the K and D ends--therefore, has been postulated (274). Indeed, cross-reactivity serologically has been detected for some K and D region products strongly supporting a basic two-region model for the end regions (274). The K and D region products are glyco-proteins (240) with m.w. of 45,000 (262), and are probably quite important in graft rejection, and also probably play some role in *MLR*.

The I region is important in the control of the immune response to a variety of specific antigens. McDevitt and Benacerraf (272) demonstrated differences in the immune response to a variety of antigens in a variety of inbred murine strains. Using branched synthetic polypeptides with a restricted range of antigenic determinants, Sela and McDevitt (294) demonstrated that certain immune responses were related to an H-2 autosomal dominant gene by the backcross technique (294). Since this demonstration, a number of other H-2 linked immune responses have been reported (281). These observations imply a high degree of polymorphism in Ir genes due either to multiple alleles or multiple Ir genes, with each individual gene determining a different response. It has also been suggested by Shreffler (262) that, in some cases, one could envision a pair of alleles at a single locus, one determining the "response" to a specific antigen and the "nonresponse" to another specific antigen, whereas the other allele expressing the converse. Lieberman *et al.*'s (295) critical observation of the combination of specific immune traits in a recombinant verified that multiple immune response genes do exist. In fact, recently, different subregions of the I region have been described (298) and it has been suggested that different subregions may code for different functions. The I-J subregion is thought to code for determinants found on suppressor T cells (307).

H-2 and Relationship to Neoplastic Susceptibility

In considering the pathogenesis of various tumors, it is essential to note that many factors have been shown to influence the incidence

with which tumors develop. Clearly, aside from immunological effects, endocrinological and environmental features modulate tumor appearance (258). Viral factors (252, 257) are important in the case of certain animal tumors. In the case of virally-related neoplasms, Lilly (252) clarified the significant relationship of Gross virus-induced leukemogenesis to the presence of the RgV-1 gene. Other tumors associated with viruses such as the B/T virus, the Mammary tumor virus, the Radiation leukemia virus are also under H-2 influence. It is possible that all such susceptibilities may be due to the same RgV-1 gene, as non-responsiveness is usually associated with the $H\text{-}2^{k}$ haplotype. Equally possible, however, are multiple immune response genes each concerned with distinct susceptibilities or resistance.

Warner has observed (258) that the mineral oil irritant induced plasmacytoma incidence in BALB/c mice is compatible with linkage to a single recessive gene. It was not firmly established in this study, however, whether H-2 linked gene influence is sufficient to account for susceptibility to plasma cell tumor development.

Recently, Sato *et al.* (259) have described a leukemia related transplantation antigen which has been termed X.1 to which certain inbred hosts appear genetically incapable of responding. Resistance to this antigen was coded by an Ir gene in the H-2 which could confer the host with the ability to respond to the X.1 antigen on leukemia cells. Experimental work has suggested that the X.1 antigen is very closely related to the murine leukemia virus MuLV. In fact, it has been shown that X.1 antigen distinguishes a subtype of MuLV distinct from passage A Gross virus.

There are undoubtedly a variety of immune defects which are related to susceptibility to neoplasia. In the murine model, for example, high tumor genotypes do demonstrate limited graft versus host reactivity (260).

In man, associations between HLA type and disease or tumor susceptibility (261) have been derived by extensive serotyping analysis. Whether these associations represent single gene effect are presently unclear.

One can conclude from these observations that immune reactivity to tumors are influenced by a variety of genes, and although certain features of resistance may be H-2 linked, other aspects of the tumor-host relationship may be under separate genetic influence. It is likely that different features of the host-tumor relationship are under discrete gene control (262).

H-2 and Cell Interaction

As already discussed, cellular cooperation between thymus-derived lymphocytes, bone marrow derived lymphocytes and macrophages has been shown to require genetic identity between the cell types. The pioneering work of Benacerraf and McDevitt (272) demonstrated that genetic factors were important in the immune response to well-defined synthetic antigens. The relationship of the genetic control of immune response and certain Ir genes has recently been analyzed (262). Shevach *et al.* (275) made the critical observation that alloantisera directed at responder guinea pig MHC loci could inhibit the activation of T lymphocytes by antigen, to which it could normally respond. Recently, Shevach *et al.* (275-276) has suggested that antigen activation of T lymphocytes

requires the interaction of different cell types. Furthermore, these are indications that there may be antigen receptors coded for on the surface of the lymphocyte, as well as another type of membrane product required for cellular interaction (278,279). These products on the cell surface which are required for genetically controlled cell interaction have been termed cell interaction products (CI), and are thought to be related to the MHC. CI, however, are separate moieties from the Ir region gene products already alluded to as being important in some antigen response (277,278). Katz *et al.* (277) have recently shown that in the murine model, successful T-B cooperation occurs when both the T and the B cell were of similar histocompatibility type. The degree of MHC necessary for such cooperation was isolated to the K end of the H-2 genome (277). Recent observations by Taussig and Munro (237,238) have suggested that the I region product on T cell has antigen specific receptor function. Moreover, Frelinger *et al.* (296) has shown that anti-Ia antisera are capable of inhibiting *in vitro* antibody response to sheep red cells. Verhulst-Schmitt and Shearer (297) have shown that Ir genes may be important in restricting cell-mediated cytolysis to hapten-modified target cells and have suggested that certain T cell interactions may be regulated by the restrictions imposed by I region gene products (297). It is apparent that certain efficient cellular interactions appear to be associated with certain I region antigens or CI antigens, all of which are part of the MHC or linked closely to it. However, in contrast to these observations are the demonstration that effective T-B cooperation can occur in the primary response across H-2 barriers.

This is particularly well seen in the chimera model of von Boehmer *et al.* (40). An explanation for such interactions is that restrictions in collaboration depend upon the antigen alteration of self components which occur in priming. The context in which T cells 'see' the antigen are related to the particular association of that antigen to the MHC determinant so as to result in the recognition of altered self. The secondary responses are optimized, therefore, when such primed animals are confronted on challenge with similarly antigen-altered self determinants (33,40,304,309). It appears that antigen must be intimately associated with I-region determinants present on macrophages to maximize T cell recognition (304,309). Thus, it would seem that the distinction of self from non-self may be the basis of many effective immune responses.

V.

RELEVANCE OF THE IMMUNE SYSTEM TO THE TUMOR-BEARING STATE

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Immunological surveillance is the hypothesis that has been used to describe the immune elimination of incipient neoplastic cells (41). Burnet (41,43) has proposed that there is a distinct evolutionary advantage for the thymus dependent adaptive immune response inasmuch as it may eliminate the cells produced through random somatic mutations or malignant defects, which represent a definite danger to the survival of the species.

The adaptive immune response appeared in the early vertebrates under the selection pressure from lethal microorganisms, parasites and possibly tumors. The phylogeny of this system reflects the change from the initial ability of invertebrates to deal with external antigens nonspecifically to the exquisite specificity of the immune system observed in higher vertebrates. The nonspecific immune response of the invertebrate was superceded and replaced by the great advantages of memory and specificity of the immune system seen in higher species. If one examines the early vertebrate, there is evidence that the cyclostomata were able to manifest a type of immune response (42) and the species that appeared later in evolution, such as teleosteae and amphibia, unequivocally had an adaptive immune response (44). It has been proposed by Burnet (41) that parasitism amongst the primitive vertebrates may have provided sufficient evolutionary pressure to develop some aspects of the primary adaptive immune response and that as vertebrates evolve, the need to counter the internal dangers of oncogenic mutation

provided the force through which the immune system of mammals evolved.

The ability to recognize changes in the cell surface falls partly within the domain of the immune system (45). With our present comprehension of the neoplastic process that affects the human species, the competency of the immune system seems to be a vital defense against uncontrolled cellular growth. Whatever factors initiate the malignant change in a cell, whether it is intrinsic to the cell or introduced by some external agent, immune recognition appears to have some influence (46) on whether or not uncontrolled neoplastic cells develop and whether or not these tumor cells are eliminated. It is apparent from a variety of epidemiological studies that cancer appears with increasing frequency at either of the two extremes of life (47,48) and, in accord with our present knowledge, it is in these extremes that the immune system is not optimally effective (47,48). It is also quite clear that if an individual is born with a severely compromised immune system (48,49), as associated with ataxia telangiectasia (48) or the Wiskott-Aldrich syndrome (48), there is an increased propensity for these individuals to develop neoplasia. Finally, as medical technology attempts to provide therapeutic solutions to conditions such as malignancy (51), transplantation (50), auto-immune diseases (52) and chronic renal diseases, the widespread use of immunosuppressive agents (51) has substantiated the relationship of immunity to neoplasia. Thus, it has been shown that as a result of treatment with immunosuppressive agent, there

may be an increased frequency of malignancy, primarily lymphomas. Consequently, medical scientists are in the undesirable situation of now having to risk inducing malignancies while attempting to cure other malignancies. To balance this negative aspect of neoplasia is the rewarding knowledge that some tumors are amenable to immunotherapy (53) and indeed some, on occasion, spontaneously regress (52) with little therapy.

Experimental evidence that the immunological system is important in dealing with malignancy was determined by examining the effects of rendering animals immunoincompetent and then examining them for an increased tumor incidence as compared to normal animals. It has been demonstrated that whole body irradiation (54), preceded by viral inoculation, led to an increased number of lymphoid leukemias in C57Bl mice as compared to the frequency of leukemias in non-irradiated or partially-irradiated controls. It has similarly been shown that treatment with ALS (55) may inhibit the immune response to tumor cells in animals, as evidenced by an increased incidence of urethrane-induced lung adenomas. This effect is thought to be due to the extensive lymphopenic action of ALS (55). Similarly, neonatal thymectomy (56) has been shown to lead to an increased incidence of tumors.

When examining isografts of methylchloranthrene-treated skin in normal and immunologically compromised hosts, Lappe (57) produced histological evidence for immune reactivity during the latent period of papilloma development. Sublethally-irradiated mice developed macroscopic lesions without any apparent microscopic immune response,

whereas normally competent mice demonstrated focal microscopic lesions exhibiting mononuclear cell infiltration. This observation strongly suggests that one of the important factors for a successful adaptive response is immune competence.

There exists a number of experimental observations contradicting the immune surveillance hypothesis. Thus, the finding that irradiated (400-500 R) C3H mice exhibit a decreased growth of subsequent tumor implants is in sharp contrast to the previous mentioned radiation effect (58). Prehn has gathered sufficient data regarding mouse mammary tumors to demonstrate that (58) where immunological manipulations should have led to an increased incidence of tumors, if surveillance were effective, there has, in fact, been a reduction in incidence. This observation has led Prehn (59,60) to postulate that surveillance operates in only certain situations and that in most cases immunostimulation has promoted tumor growth, *i.e.*, Prehn considered the possibility that a low level of immunity to tumor actually enhances tumor growth. Other evidence which questions the surveillance concept is the observation that neoplasms which develop "spontaneously" in mouse cells in tissue culture, or in the protective confines of diffusion chambers, have generally been shown to possess low levels of antigenicity (59). This is not compatible with the *a priori* assumption that most spontaneous tumors should be highly antigenic. If surveillance were functional, these spontaneous, highly antigenic tumors would be expected to be rapidly eliminated leaving the poorly antigenic tumors to account for the observed neoplastic disease (58). This is not sufficient evidence to invalidate

the surveillance hypothesis since, in the context of the evidence reviewed so far in this thesis, one may postulate that a suppressor T cell population may account for this observation. Furthermore, it has also been shown that tumor latency but not tumor incidence is related to immunological competence (58). This fact does suggest that active selective pressure is exerted immunologically on developing tumors. Recently, the observation by Stutman (61) that nude mice are highly susceptible to polyoma virus oncogenesis, at ages when immunologically normal controls are resistant, suggests that surveillance is important in resistance to tumor induction. Moreover, Stutman (61) has shown that thymic transplants conferred on nude mice some resistance to tumors (61). However, the recent extensive studies by Rygaard and Poulsen (310) have shown that there are relatively few, if any, cases of spontaneous malignant tumors when nude mice were observed under conditions of a well-controlled environment. It was concluded that the microbiological milieu might be a greater force for the maintenance of cell-mediated immunity during evolution than the pressure of spontaneous tumor generation.

VI.

IMMUNOLOGIC ASPECTS OF HOST-TUMOR INTERACTIONS

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Tumor Cell Antigens

Carcinogen-Induced Tumors

The idea that tumors express antigens which can evoke an immune response even though this response may be weak, represents a basic concept of tumor immunology. Within the context of this thesis, such antigens will be referred to as tumor antigens. The first clear demonstration of the specific antigenicity of certain experimental tumors was made by Foley (62). Thus, by ligating chemically-induced sarcomas he showed that the animals were rendered resistant to the subsequent implantation of the same tumor. It was also shown that spontaneous mammary tumors, when treated in the same manner, did not induce immunity. The confirmation of Foley's work by Prehn and Main (63) included a series of controls that demonstrated that the immunity so induced was specific for the tumor and could not be attributed to any residual genetic disparity among the inbred animals used. The more definitive work of Klein and colleagues (64) showed that the primary host, after excision of the tumor, could be made resistant to further challenges with autologous tumor, by the repeated immunization of heavily irradiated cells of the same tumor.

It has been generally accepted that the antigens of tumors induced by the application of chemical carcinogens to other syngeneic individuals are unique for each of the tumors (56,66), *i.e.*, the antigens show no cross-reactivity (66), and resistance to rechallenge is specific only for the particular immunizing tumor. In one series of

experiments, Basembrio (67) established that there was no cross-immunization among ten methylchloranthrene-induced sarcomas. Recently, however, this generalization has been challenged by Foerbes *et al.* (71) who demonstrated both *in vitro* and *in vivo* cross-reactivity among soluble tumor antigens isolated with 3MKCl from several methylchloranthrene-induced tumors. By immunization with the solubilized tumor antigens, they showed that transplantation immunity *in vivo* was evoked by shared or cross-reacing antigens. Therefore, it would appear that the number of antigens capable of being induced by methylchloranthrene is finite.

With respect to the antigenicity of the tumor, Haywood *et al.* (72) suggested that there existed an apparent inverse relationship to H-2 antigenicity expressed on the same cells, in that highly immunogenic tumors were those that have quantitatively less H-2 antigen on their surface and vice versa. This idea suggests a definite relationship to cell surface dynamics and implies an H-2 genetic relationship of the tumor-associated antigem. Fujimoto *et al.* (212) have clearly demonstrated that tumor antigen may be physically linked to H-2 antigens. It is conceivable that H-2 membrane proteins are modified by tumor antigens, so as to provide a suitable signal as altered self to the surveillance mechanism (203,).

Foetal Antigens

There are certain similarities between the rapid growth seen in the embryonic development and the uncontrolled growth seen in malignancy. It was demonstrated that α fetoprotein macromolecules found in an embryo were also present in hepatoma tumor tissue (73). Xenoantisera were raised to either a tumor or an embryonic extract and, after suitable absorptions, it was shown that the antibody recognized antigens common to both the tumor and embryo which were absent from normal adult tissue. Similarly, Gold and Freedman (74) reported a carcinoembryonic antigen (CEA) which was present in adenocarcinomas of the human digestive tract as well as in human foetuses, after conception and that it was absent in other benign tumors. The complete chemical structure of CEA has, as yet, not been established (75), but it has been shown to be a glycoprotein (75) and it may have more than one distinct antigenic determinant. However, recent work has shown that CEA may be present in other non-gastrointestinal malignancies and even in some benign conditions (76). The correlation between the appearance of embryonic antigens and the development of neoplasms implies that tumors may arise as a result of derepression of certain genes that are normally quiescent in adult life (73).

Viral Antigens and Relation to Tumors Induced by Virus

There are two main types of oncogenic viruses: the oncornavirus which are RNA viruses as exemplified by the murine leukemia virus (MuLV), and DNA viruses as exemplified by the polyoma virus. It has been reported by Sjögren (77) that different

tumors induced by the polyoma virus share a common antigenic component distinct from other tumor-specific antigens. Some degree of immunity can be demonstrated with respect to challenge with isogenic polyoma tumors and can be induced in adult mice by infection with polyoma virus. The persistence of antigens related to the causative virus has been observed in tumor cells (78), in which little or no infectious virus can be demonstrated (79). Huebner (269) has shown that the sera of hamsters bearing either primary or transplanted tumors, induced by adenovirus type 12 or 18, contained antibodies that reacted in the complement fixation test with the corresponding type specific adenovirus antigens. The antigen (269) could be obtained from tumor tissues which contained no infectious virus, and also from cultures of human tissues infected with the virus. Therefore, it would appear that viral antigens persist in virus-induced tumors, independent of demonstrable virus.

There are also other antigenic systems associated with viral tumors such as the Gross (78) leukemia antigen (G antigen), the Friend virus leukemia system antigen which is related to the Rauscher virus (79), and others such as the TL antigen (80), a leukemia-specific antigen, present in the thymus cells of mice with leukemias.

Aoki (81) has recently used immuno-electronmicroscopy of mouse cells which had been infected with MuLV to demonstrate complete or incomplete virions produced by cells bearing Ly-A, Ly-B and H-2 alloantigens. Of these antigens, only the H-2K and θ antigens were present on the virion surface. In most cases, these virions, however,

carry little discernible H-2 antigens and no Ly antigens.

Recently, Grant *et al.* (82) have further demonstrated that culture cells of methylchloranthrene-induced sarcomas produced infectious MuLV. It was also shown that antisera against MuLV could lyse the tumor cells, implying that viral structural components could exist on cell surface independent of virus production. Clearly then, on a variety of murine tumor cells, there exist antigens, some unique to the tumors (79) but others shared by closely-related or virally-induced tumors.

It is apparent with respect to the MuLV and Maloney sarcoma virus (MSV) that tumor cells shed virus, and cells which apparently are not producing virions may have surface antigens which cross-react with viral products (81). Similar information about the determinants of the Gross antigen is still not entirely clear. It is necessary that common viral antigens be defined property, so as to elucidate their role in eliciting immunity, and to use them as markers for virus-induced tumors. The observation that tumor viruses may immunize in certain situations rather than generate a tumor (77) may be exploited in the future for immunotherapeutic intervention or prophylaxis.

VII.

EFFECTOR MECHANISMS IN DESTRUCTION OF TUMOR CELLS

The macrophage, according to Krahenbuhl and Remington (85), does have certain discriminatory abilities as judged by the observation that the activated macrophages produced only slight destruction of embryo fibroblasts which had not undergone spontaneous transformation. These macrophages were not cytotoxic for normal 3T3 fibroblasts. This suggests that surface membrane alterations, whether biochemical or topographical, may be important in target cell recognition and destruction by macrophage. Holterman (86) has also reported that peritoneal macrophages of PPD-stimulated rats produced cell destruction of tumor cells; yet, apparently little or no cytotoxicity was exerted on syngeneic or allogeneic second passage normal kidney cells.

Evans and Alexander (87) suggested that syngeneic peritoneal macrophages from mice immunized against L5178Y lymphoma cells exert a cytotoxic effect on these cells *in vitro*, and that normal syngeneic macrophages may be rendered cytotoxic towards L5178Y cells by incubating these macrophages with immune spleen cells for 24 hours (89). Still another method for obtaining "armed" macrophages, which are highly cytotoxic, is to expose them to the supernatant collected after incubation of immune lymphoid cells with specific antigen (90). Alexander has further shown that exposure of immune reactive cells to specific antigen renders the cells nonspecifically cytotoxic. The whole process apparently represents a two-step procedure (87-91), *i.e.*, an activation step, which is antigen-specific and subsequent killing of target cells which may be nonspecific.

Alexander and Evans have also shown that doubled-stranded RNA and endotoxin can confer cytotoxic capacity on macrophages *in vitro* (88). However, the mechanism for this activation of macrophages is obscure. Alexander speculates that "specific" macrophages arise early (seven days after tumor implant) and that they may later (> 15 days) act "non-specifically". These "activated" macrophages show an increased tendency to phagocytose (87-90) and spread (91). Although it has been shown lately that macrophages can limit the proliferative capacity of tumor cells *in vitro*, there is no evidence that they may act in a similar manner *in vivo* and, therefore, the significance of these observations remains to be established. Another mechanism by which macrophage become armed might be via the Fc receptor thought to exist on its surface (110,111).

Thymus-Derived Cells as Effectors

Early observations in tumor immunology demonstrated that rejection of tumors and grafts could readily be transferred from sensitized donors to normal recipients with lymphocytes but not with sera. It is apparent from the work of Lamon *et al.* (92) that *in vitro* activity against syngeneic (irradiated) methylchloranthrene-induced tumor cells, as measured by the microcytotoxicity assay, was mediated by T cells as well as by non-T cell participants.

Plata *et al.* (102) have recently investigated the killing of syngeneic tumor cells from MSV tumor-bearing animals throughout various stages of tumor growth and regression. It is apparent from the work of Holden *et al.* (311) and Röllinghoff and Wagner (99) that thymus-dependent (T) cells are necessary for syngeneic tumor lysis in certain stages of tumor bearing in the animals studied, in both *in vitro* and *in vivo* assays.

Tucker *et al.* (93) studied the transplantable lymphoma (C58NT)D induced by the Gross virus in W/Fu rats. Treatment of spleen cells obtained from rats immunized with this syngeneic tumor, with anti-thymocyte serum (ATS), specifically decreased their cytotoxic capacity as judged by the ^{51}Cr release assay, indicating that the major effector cell was a thymus-derived lymphocyte.

Brunner and Cerottini (211) have demonstrated that the spleens of DBA/2 mice which had been injected intraperitoneally with irradiated syngeniec L5178Y lymphoma cells and which were challenged with increasing numbers of viable L5178Y cells, contained lymphocytes which were specifically cytotoxic for the lymphoma cells but did not destroy the syngeneic P815 cells. Hence, these results indicate that sensitized lymphocytes were readily demonstrable in the spleens, as assessed by a growth inhibition assay and that the observed target cell destruction was specific. Shortman *et al.* (94) studied the progenitors of cytotoxic lymphocytes in terms of density distribution. They reported that the buoyant density of the CL population developing in the spleen of immunized animals showed progressive changes with time, *i.e.*, in the early stage, cytotoxic lymphocytes were large and had a relatively low density and with time their density increased and they finally approached the size of small lymphocytes.

Berke, Sullivan and Amos (96) have shown that effector lymphocytes can interact repeatedly with tumor cells, thereby accounting for the significant damage observed by a small specific effector population.

Tevethia *et al.* (95) have demonstrated that the cellular response to SV40 tumor cells *in vivo* in syngeneic mice is mediated by θ positive thymus-derived lymphocytes. Relevant to this is the demonstration by Rouse (100,101) that in syngeneic plasma cell tumor studies, T cells directly inhibit tumor growth. It is also worthwhile mentioning the recent demonstration that thymus-derived lymphocytes are also the effector cells in the secondary response to tumor antigen (210) in a syngeneic spleen.

In many studies, the specificity of cytolytic T lymphocytes (CTL) is directed to certain modified products of the major histocompatibility complex, and in the mouse which has been investigated extensively it is, in particular, the H-2K and/or the H-2D loci (303).

CTL responses can be raised to syngeneic cells as well, if such cells are virally-infected or chemically-modified. In this case, the specificity of CTL, although encompassing the relevant viral or chemical modifier, is nevertheless again directed at the H-2K and/or H-2D loci (304,305). It has been proposed that CTL clones have receptors specific for the slight modifications of the products of the autologous H-2K or H-2D loci. This may extend to the case of tumor cell cytolysis, as it has been clearly shown that alloantisera directed to H-2K and/or H-2D specificities inhibit tumor cell destruction (203).

From all these data, the significance of the thymus-derived cell in relation to the elimination of neoplastic cells appears well

substantiated (97-99). Therefore, it is justified to consider that T cells are an integral part of the immune response to tumor antigen (101,102).

B Cell as an Effector

As is generally accepted, antibody-producing cells are important for the normal immune response to a variety of antigenic stimuli. Moreover, as shown recently, Shin *et al.* (103), lymphocytes not bearing the θ antigen, *i.e.*, presumably B cells, may be also effective in inducing the antibody-mediated suppression of lymphomas. Lamon *et al.* (104) have also found that thymic independent lymphocytes are effector cells for the MSV tumors in BALB/c mice.

Recent work by O'Toole *et al.* (105) demonstrated that human patients with transitional cell carcinoma possess cytotoxic effector cells in the peripheral blood with specific activity for the tumor and showed that removal of cells bearing the Fc receptor and surface Ig depleted the lymphocyte preparation of cytotoxic activity. Furthermore, these workers demonstrated that the residual cells, after removal of T cells by the E-Rosette technique, were still cytotoxic and concluded that the effector cells for this tumor were not thymus-derived and depended on the presence of B cells. Schirrmacher *et al.* (106) have shown that specific antibody may be the mediator of cytotoxicity when soluble antigen coats target cells. It was shown that antibody may confer specificity to the cytolytic reaction ultimately produced by other effector cells belonging to a different class (106).

This finding that antibody-coated target cells can be destroyed by nonimmune lymphoid cells has been confirmed by a number of workers (107,108) and shown to be thymic independent. The possibility that B cells are important was suggested by van Boxel *et al.* (109) who showed that pretreatment of spleen lymphocytes with anti-κ serum and C' eliminated the effector cell activity against antibody-coated target cells. However, recently, Greenberg *et al.* (110) suggested that the antibody-dependent cytotoxic effector cell in nonimmune spleen cells is, in fact, a nonphagocytic monocyte (111). It appears, moreover, that there exist species differences, in that the effector cell receptor may differ in one species as compared to another (113). Furthermore, Zighelboim (112) has shown that the EL-4 tumor cell appropriately treated with anti-tumor antibody can be lysed by adherent syngeneic cells. Although the cytotoxicity is exerted on the target cell coated with the antibody, the mechanism responsible for cell lysis appear to require cell-to-cell contact because no evidence for remotely acting cytotoxic factors during lysis was found (112,113). There is increasing evidence that non-T cell effector mechanisms, which have been described as natural killer systems exist (306). These natural killers obtained from non-immunized spleens of a wide variety of strains, operates through an H-2 influence and carries a unique surface by antigen detected by a new anti-Ly 1,2 sera. From the survey of these studies, it may be suggested that different effector mechanisms operate in

different systems and that different effector cells may be functional at different times in the immune response to tumor antigen.

VIII.

HOST-TUMOR INTERACTIONS

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Tumor Promotion by Antibody or Antigens

As reviewed earlier, it is apparent that antigens exist on tumor cells and can be shown, with proper manipulations, to be immunogenic. One of the central themes of tumor immunology is to elucidate the reason for the failure of these antigens to elicit an effective vigorous immune response, which may be attributable to some unique feature of the tumor-host relationship.

The immunological facilitation or enhancement of growth of antigenic cells in immunocompetent hosts has been ascribed to various "blocking factors" present in the sera of tumor-bearing hosts. Thus, Gorer and Kaliss (184) demonstrated that passively administered alloantisera could either inhibit or enhance the growth of various tumors in murine hosts and concluded that the tumor, as a consequence of contact with antibody, may have undergone some modulation permitting it to escape the immune response of the host. Klein and Sjögren (185) reported that, in a murine system utilizing methylchloranthrene-induced tumors of known genotypes, isoantiserum enhanced the growth of the tumors in genetically incompatible systems, whereas in isologous systems it had no effect. It is significant that these same authors showed further that lymph node cells obtained from the draining axillary or inguinal nodes of preimmunized homologous hosts were inhibitory to tumor growth in both homologous and isologous systems. Möller (186) examined the effects of immune syngeneic sera on syngeneic methylchloranthrene-induced mouse sarcomas and concluded

that antibodies directed against tumor-specific antigens exist in tumor-bearing mice and can be demonstrated by their capacity to enhance or inhibit tumor growth in syngeneic recipients.

Hellström developed the colony inhibition technique for studying cell-mediated reactions to tumor antigens. This technique measures the effect of immune lymphocytes on the development of colonies of tumor cells *in vitro*. Lymphocytes obtained from tumor immunized hosts cause inhibition of colony formation. The micro-cytotoxicity assay has also been used to determine the immune capacity of lymphocytes from tumor-bearing animals as judged by the ability to kill tumor cells or inhibit their division. It has been shown that while cellular immunity may be demonstrable *in vitro*, it cannot be adequately demonstrated *in vivo* in tumor-bearing hosts. Hellström *et al.* (187) were able to show, with their colony inhibition test, that sera from tumor-bearing animals obtained at the time of tumor removal and explantation could abrogate the inhibitory effect of autochthonous lymph node cells on the growth of tumor. It was also noted that sera taken prior to the tumors becoming palpable had no effect on inhibition of the lymph node cell. Interestingly, these same authors showed that splenectomized animals had significantly less blocking activity in their sera than sham operated controls. Hellström originally suggested that anti-bodies were responsible for this effect (188). However, more recently, they reported that the situation was more complex and that

inhibition of colony formation may have been due to antigen-antibody complexes (195). Moreover, dissociation of these complexes and separation of antibodies from their antigenic moieties, at low pH, resulted in a loss of blocking activity of the sera. This blocking activity could be recovered by recombination of the two fractions.

Bloom and Hildemann (189) also studied a methylchloranthrene-induced sarcoma in C57B1/10 mice and showed that cell-mediated immunity is associated with resistance to tumor during a critical period of 15 days following challenge. Furthermore, IgM antibody from resistant mice was capable of curtailing tumor growth *in vivo*. However, the 7S fraction obtained from tumor-bearing hosts, with actively growing tumors, showed detectable tumor enhancing activity *in vitro* and *in vivo*.

In a more recent report, Hildemann and Bloom (190) showed that preimmunization with viable sarcoma cells, followed by excision, in some cases could lead to enhanced growth of a subsequent challenge, compared to the growth rate of the primary tumor. Further, in these experiments, when the primary tumor was not excised, secondary tumors grew at essentially the same rate as seen when the primary was removed, as if to suggest active enhancement of the secondary tumor by the primary. The authors concluded that antigen-antibody complexes may account for the inhibition observed, but an additional regulatory effect exerted by immunocytes was also considered.

Eustace and Irvin (191), using an EL-4 leukosis in a murine model, showed that removal of the 7S γ 2a alloantisera fraction resulted in loss of immune inhibitory activity; as evidenced by failure of animals receiving this antisera to show increased tumor growth. The exact moiety was not precisely defined. Recently, Irvin and Eustace (192) have also demonstrated that tumor EL-4 allografts could be either promoted or inhibited with respect to growth of tumor, by allograft sensitized regional lymph nodes, the effect depending on certain critical time intervals. It has been proposed by Baldwin (193) that tumor antigens related to the aminoazo-induced tumors in syngeneic rats may induce "blocking" by binding of various serum factors to neoantigens expressed upon tumor cells, thus preventing cellular recognition by sensitized effector cells. Inhibition of effector cell reactivity may also occur, due to circulating immune complexes, as exemplified by the observation that post-excision serum could reverse blocking as measured by cytotoxicity *in vitro*. Furthermore, by monitoring the level of D23 hepatoma-specific antigen, it was found that circulating tumor antigen appears early and as immunity develops, there is decrease of tumor antigens as immune complexes become detectable and,finally, free antibody becomes apparent in addition to immune complexes in the late stage of tumor growth.

Alexander (202) has recently described soluble substances in the m.w. range of 50,000 daltons in the blood, lymph and urine in both tumor-bearing humans and rats, which by radioimmunoassay was

indistinguishable from tumor antigen. Furthermore, Alexander speculated that antigen shedding may promote metastasis.

Recently, Germain *et al.* (203) have shown that certain anti-H-2 alloantisera specific for the target tumor cells can block lysis of those target cells mediated by syngeneic tumor-specific cytotoxic lymphocytes. This study suggests that tumor-associated antigens best able to elicit cytotoxic lymphocyte responses may represent altered or modified H-2 antigens.

That tumor antigen itself is responsible for arrest of the immune response has been suggested by Alexander and others (198,202) who proposed that, in many cases, the initial escape from immunological elimination could be due to tolerance to tumor antigen. By studying the immunoblast response in draining lymph nodes, Alexander (202) claimed to have shown that in the presence of an actively growing tumor, the essential cellular response needed, if there is to be immunity, fails to occur, in that immunoblasts are not discharged into the circulation. The paralysis is apparently reversible within 24 hours of surgical removal of the tumor and the effect is, furthermore, specific inasmuch as it is confined only to the response to the relevant tumor antigen. Moreover, Bansal (196) has apparently detected unblocking activity in the sera of rats which had tumors removed. When tumor challenged rats were given unblocking serum, a high percentage showed some regression of the tumor. It was proposed that unblocking antibody might interact with soluble antigen-antibody complexes and lead to an

interaction of the unblocking antibody and the antigen. Alternately, there may be a conversion of the complex from one of the antigen excess to one of the antibody excess (194,196).

It is entirely relevant to this discussion to relate the observation of Plata *et al.* (197). Briefly, T and non-T cell suspensions were prepared from the spleens of C57Bl/6 mice immunized with MSV and simultaneously assayed in the chromium release tests of syngeneic lymphoma cells and in a microcytotoxicity assay against adherent cultured MSV tumor cells. T and non-T cells were active in the microcytotoxicity assay but only T cells were active in the ^{51}Cr-release. Also serum from tumor-bearing mice or alternately soluble antigen could disrupt the microcytotoxicity growth inhibition but had no apparent effect on T cell-mediated cytolysis in the Chromium Release Assay (CRA).

Therefore, some of the effects reported in the literature may reflect different mechanisms of target cell lysis operative in different assay systems.

Experimental Tumor Models Suggestive of Immunoregulatory Mechanisms in Tumor-Bearing Hosts

Mikulska *et al.* (199) reported that recipients of a benzopyrene-induced fibrosarcoma could be protected from tumor growth by mixing syngeneic spleen cells with tumor cells *in vitro* prior to injection into the recipients. Autochthonous spleen cells taken three weeks after the excision of the tumor, when mixed with the tumor cells, iduced rejection of this tumor inocula. However, spleen cells taken

at the time of the excision of the primary tumor could not induce an immune response when transferred to syngeneic recipients.

Irvin and Eustace (191,192,200) showed that adoptively transferred lymph node cells from mice bearing tumor allografts could, if taken on day 12 or 14, enhance tumor growth in syngeneic tumor-bearing recipients. These authors concluded that this enhancement was due to the production of 7S γ1 globulin antibody.

Gershon (201) showed that in allotransplantable lymphoma systems, spleen cells were suppressive during the first seven days after tumor inoculation, and apparently could promote a tumor growth. This was reversible inasmuch as splenectomy within the first seven days retarded the tumor growth. Okubo *et al.* (204) have recently shown that when graft-versus-host reactions were induced in (BALB/c x C57Bl/6)F_1 hybrids by intravenous transfer of parental spleen cells followed subsequently by a methychloranthrene tumor inoculum of 1.25 x 10^5 cells s.c., the growth of such tumors was markedly accelerated. There was no circulating antibody functional in this reaction. It was postulated that there might be destruction of T killer cells or a potentiation of suppressor cells. Elkins (205) have also indicated that the partial tolerance observed after adoptive abrogation of transplantable tolerance in Lewis rat chimeras might be mediated by suppressor cells.

Kilburn *et al.* (206) have recently shown that a lymphocyte with characteristics of an activated B cell may act as a suppressor cell in a MSV-induced tumor system.

Umiel and Trainin (207) have shown that lymphocytes of tumor-bearing animals could stimulate the growth of 3LL tumor in syngeneic C57BL/6 mice and supported that this reflected active suppression by lymphocytes of the immunological response to tumor.

Recently, Gershon *et al.* (208) have demonstrated that there exists a thymic dependence of the ability of antibody and tumor cells, acting as complexes, to suppress the binding of tumor cells to macrophages. This suggests a thymic influence in the modulation of the immune response to tumor cells. It has,furthermore, been recently found (209) that a deficiency of T lymphocytes might lead to reduced tumor growth of carcinogen-induced tumors in mice.

These data imply that in several tumor systems, lymphocytes and predominantly thymus-derived lymphocytes can modulate the tumor handling by the host. It is the intent of this study to clarify the regulation of the immune response to tumor antigen by thymus-derived lymphocytes. The concept that the tumor antigen-host interaction might lead to an abnormal regulatory response of the immune system prompted the present study.

IX.

SCOPE OF THE PRESENT STUDY

SCOPE OF THE PRESENT STUDY

The scope of the present work included the examination of three aspects of host-tumor relationship. In the first part of the work, the question that was addressed was whether there existed a cellular basis for the observed quiescence of cytotoxic effector potential in the tumor-bearing host.

For some time in tumor immunology, there has been a central unresolved problem dealing with the explanation of why tumors are not rejected in the same manner as foreign grafts. Since the work of Foley (62) and Prehn and Main (63) established conclusively that tumor antigens exist on the tumor cell and can evoke an immunological response, it has been unclear why such responses are not effective in the primary tumor-bearing host. A great deal of investigation necessarily has focused on this problem.

Hellström *et al.* (125) should be credited with focusing the problem to the responses that the host did direct to the tumor. The most important contribution of these workers is the concept that arose in which "blocking" factors were said to be found in the sera of tumor-bearing mice and shown capable of blocking various tests thought to reflect T cell-mediated cytotoxicity (196,125).

Problems of identification of specific sera-borne factors has led to ambiguous answers as to what really mediates the block (196); although soluble tumor antigens clearly are important. Nevertheless, in many cases, blocking factors are not apparent (229); and other suppressive mechanisms must be operative.

To define whether a cellular suppressive mechanism capable of obviating effector responses existed, case I investigated a tumor system in which the murine host could be made immune to the tumor. By this means it would be possible to gauge changes in the effector capacity by the adoptive transfer of various cell types, sera or other material.

It was decided that an appropriate model would be using a methylchloranthrene-induced fibrosarcoma. The S1509a syngeneic to A/J ($H\text{-}2^a$) was, therefore, used. The rationale for such a tumor system can best be understood because of the relative simplicity by which animals carrying the tumor can be made immune to it. This state of immunity can be achieved by the complete surgical excision of the tumor and after this subsequent challenges of tumor cells were rejected consistently. Furthermore, since this tumor grew subcutaneously as well as intraperitoneally (i.p.), it could be easily adapted to *in vitro* culture. This *in vitro* cultured tumor line could be replaced at regular intervals from *in vivo* (i.p.) maintained tumor, and could also be used appropriately in many *in vitro* assays of tumor immunity, such as antibody-mediated cytotoxicity.

Concurrent with this investigation into cellular suppressive mechanisms, was the expansive experimentation elsewhere (reviewed in 230) that demonstrated a negative regulatory cell operative in a number of antibody systems. In many cases, these regulatory cells were found to be antigen-activated thymus-derived cells (175).

Because of these observations, it was thought useful to investigate the effect of cells obtained from a variety of organs of animals bearing the tumor, when transferred to animals with immunity to the tumor. The animals bearing the growing tumor would be constantly exposed to tumor antigens and it was reasoned that the cells in different organs might encounter the antigens and become specifically activated to modulate the host directed response to tumor. It was clear that tumor immune animals rejected tumor inocula (*e.g.*, 10^6 tumor cells) consistantly in a fixed period of time, and with a well-defined pattern that varied little between immune mice. These observations allowed the relatively simple experiments in which immune animals could be challenged with a tumor inocula and also receive adoptively transferred cells, etc. Alteration of cytolytic effector mechanisms responsible for tumor rejection could be followed. The results of these experiments dealing with the existence, isolation and characterization of suppressor T cells operative in the tumor system are disclosed in Chapter I and II of the thesis. In these chapters, I shall present experimental data which verify that regulatory cells arise in a tumor bearing host shortly after exposure to tumor antigen. Such cells have been characterized and will be shown to be thymus-derived (T) cells as well as being cortisone sensitive, light in density, and capable of acting in a specific manner to limit the cytolytic response to tumor cells.

The second major question that was probed in the context of the host-tumor relationship was whether a subcellular product mediated

the suppression of the immunological response. I took advantage of the technique of cell rupture, and examined the biological activity of the membrane fragments thereby produced. This series of experiments which are described in Chapter III reveal that an antigen-specific cell-surface product is responsible for the modulation of the immune response to tumor. In a variety of experiments, it will be shown that such membrane factors appear to be the gene products of the K end of the H-2 major histocompatibility complex.

A large number of investigators (168,175) have described antigen-specific membrane-derived factors operative in a variety of systems. In most situations that have been systematically probed, the suppressor cells are of the Ly $2,3^{+}$ phenotype (177,302) and the suppressor factor carries antigenic determinants derived from the I-J subregion of the MHC (176). These studies imply that the ontogeny of antigen-specific factors are related to a certain differentiated lymphocyte class and, further, that MHC region genetically defined membrane products may have function related to the region that coded for it.

The relevant biological significance and the importance of this observation as it relates to the tumor-bearing state are discussed in Chapter III in great detail. The particular characteristics of the suppressor factor are also clarified in this chapter.

In the further definition of the factor, and from observations that suppressor cells can act at the effector cytolytic stage of tumor rejection, it was thought that a scheme of the molecular

interactions of antigen-specific membrane factors and tumor antigen could be defined. The scheme of action as outlined in Chapter III interpret the data presented in the context of tumor antigen-suppressor factor complexes interacting with the relevant effector cell or precursor of that cell and either preempting differentiation or terminating the effector cell activity directly.

The third facet of study focused on the question of surveillance as it applies to the tumor state. Surveillance as described by Burnet (41) implies that the thymic dependent response is necessary for the elimination of incipient oncogenic events. Because of this concern, the question of surveillance was studied with an attempt to discern whether the thymic-dependent system becomes operative only after tumor growth was well established. It was proposed that early in ever-changing history of the tumor, non-thymic dependent effector processes might predominate, and that after tumor is established (*i.e.*, rapidly growing) suppressor T cells become operative. This question was examined in Chapter IV and V.

In these chapters, it will be shown that at a very early period of the tumor host relationship when the tumor is small ($< 10^3$ cells) non-T cell processes function as effector mechanism, and are not amenable to the suppressor T cell modulation seen with larger progressively growing tumors ($> 10^6$ cells). The most important observation of these studies is the separation of the effector regulatory systems operative at different 'moments' in the history of tumor growth. Although not amenable to modulation by suppressor T cells, it was found that solubilized tumor membrane given at the

same time as the small tumor inocula, could consistently 'block' the primary effector response. The ability to modulate the primary cytolytic response by a different mode than regulatory cells is thought therefore to reflect a different biology of the cell type involved. Furthermore, in Chapter V, the nature of the primary response was further studied to establish the precise characteristics of the tumor's growth which dictated whether and when suppressor cells were generated.

The present study has focused on three areas fundamental to our understanding of the host tumor relationship. These areas are (1) the identification of regulatory cells in the tumor-bearing host and their characteristics; (2) the nature and genetic relationship of the T-cell membrane product responsible for suppression of the immune cytolytic effector response and (3) the characteristics of biological responses to small primary inocula of tumor and the difference in these responses from those of murine hosts with progressively growing large tumors.

The biology of the host-tumor relationship is complex and at this moment beyond our complete comprehension. The following chapters have hopefully shed some light on this vast and difficult subject. Each Chapter contains references to material found within it, and as such the references will not correspond to those in the bibliography which relate to the introduction and discussion.

X.

STUDY OF THE REGULATION OF THE IMMUNE RESPONSE TO TUMOR ANTIGEN

THE FOLLOWING FIVE CHAPTERS REPRESENT THE EXPERIMENTAL BASIS FOR THE THESIS.

CHAPTER 1

REGULATION OF THE IMMUNE RESPONSE TO TUMOR ANTIGENS. IMMUNOSUPPRESSOR T CELLS IN TUMOR-BEARING HOSTS

The existence of tumor antigens on malignant cells has been demonstrated with increasing frequency in recent years. These antigens are capable of eliciting a cell-mediated immunologic response, which is manifested by the rejection of tumor cells implanted in syngeneic animals after the complete excision of the primary tumor, especially in the case of tumors induced by a chemical carcinogen such as methylcholanthrene (1-3). However, the rampant growth of the antigenic tumors in primary hosts, in spite of an apparently concomitant immune response, has been attributed to "blocking" factors in serum of tumor-bearing animals (TBA) that inhibit the destruction of tumor cells by effector lymphocytes (4,5); the blocking effect has been imputed to "blocking" antibodies (6) or to free antigen(s) shed by tumor cells or to the corresponding antigen-antibody complexes (7,8).

Whereas the antibody response to ordinary antigens, *e.g.*, sheep erythrocytes (9,10) and several hapten-carrier conjugates (11,12), was shown to be the result of the synergistic cellular interaction between T (thymus-derived) and B (bursa equivalent) cells, the cell-mediated immune response (13-16) in graft-vs-host (GVH) reactions in allograft rejection has been recently shown to be the result of the synergistic interaction between two subpopulations of T cells. Moreover "negative" cellular interactions causing suppression of the immune response have been recently discovered in relation to both the humoral (17-22)

and the cellular (23-25) manifestations of the immune response; these negative effects have been attributed to "suppressor" T cells possessing the ability of reducing the humoral or cellular immune response on interaction with the appropriate immunocompetent cells.

The present investigation has been prompted by the belief that the apparent ineffectiveness of the immune response of tumor-bearing hosts in rejecting antigenic tumors might be due to the negative cellular interactions mediated by "suppressor" T cells. Indeed, the experimental findings reported here provide experimental evidence for the existence of specific "immunosuppressor" T cells in hosts bearing the antigenic methylcholanthrene-induced Sarcoma 1509a, which is transplantable in A/Jax mice.

Materials and Methods

Animals. Two to 3-month old mice of the inbred A/Jax strain (A/J), purchased from Jackson Laboratory, Bar Harbor, Maine, were used throughout this study.

Characteristics of tumors. The sarcoma 1509a induced by methylcholanthrene in A/J mice was kindly donated by Dr. P. Wright of the National Institutes of Health, Bethesda. For propagation of the tumor, 10^5 sarcoma cells grown in the peritoneum of tumor-bearing donors and harvested in the ascitic fluid were transferred i.p. into 3-month old mice or were cultured in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.), which was supplemented with 5% fetal calf serum (FCS). Cells harvested after

three *in vitro* subcultures were inoculated subcutaneously (s.c.) into normal A/J mice at a dose of 10^4 cells and found capable of growing in these hosts and killing them within 2 months. For a successful s.c. transfer of the tumor, a minimal dose of 10^4 cells was necessary.

Lymphoma L1117 and the anaplastic Carcinoma 1509A which are syngeneic but antigenically unrelated to the Sarcoma 1509a were used in control experiments. The lymphoma L1117, discovered in this laboratory, had been shown to be derived from thymus cells of A/J mice; the anaplastic Carcinoma 1509A, which is a spontaneous mammary carcinoma in A/J mice, was purchased from Jackson Laboratory. Both lines of these unrelated tumor cells were maintained by i.p. transfer into 3- to 5-month old A/J mice *in vivo* and by culture in RPMI 1640 medium supplemented with 5% FCS.

Procedure of immunization. For immunization of the 1509a tumor, A/J mice received s.c. 10^6 cells of the sarcoma in the center of the back and the tumors were completely excised surgically 1 week later. After several subsequent injections of tumor cells, beginning 2 weeks after the initial excision of the tumor, the animals developed strong immunity as demonstrated by the complete rejection of an inoculum of 1509a cells even at a dose of 10^8 cells.

Preparation of thymus and spleen cell suspensions. Thymuses and spleens from 1509a-bearing mice were carefully removed 1 week after inoculation of 10^6 tumor cells and gently pressed in chilled Hanks' solution with glass grinders to release cells from fibrous

tissues. The cells were passed through a No. 150 mesh platinum screen and washed three times with cold Hanks' solution.

Experimental design for the demonstration of immunosuppressor cells. The *in vivo* assay system developed for the demonstration of the presence of suppressor cells in thymuses or spleens of tumor-bearing mice consists simply in establishing visually or histologically the degree of inhibition of tumor rejection in syngeneic immune animals by i.v. transfer of washed thymus or spleen cells of TBA. Thus, tumor growth was determined i) macroscopically with Vernier calipers in terms of two diameters at right angles by at least two investigators independently, after depilatation of the tumor sites with a chemical hair remover (Neet) (since the tumors were relatively flat in immune animals) and ii) histologically under the light microscope after staining of the appropriate tissue sections with hematoxylin and eosin. Immune mice receiving thymus and spleen cells from normal A/J mice or from mice bearing unrelated tumors served as controls.

Tumors were extirpated from animals in experimental and control groups 3,5,7 and 9 days after transfer of lymphocytes from TBA and fixed with 10% formalin for histologic examination. At least five syngeneic mice were used for each experiment, and each experiment was performed at least in duplicate. The results were pooled so that each group consisted of 10 animals. The statistical significance of the results was calculated by the Student t-test; the

computed standard errors are indicated in the appropriate figures and tables.

AKR anti-θ C_3H serum. C_3H serum was elicited in AKR/J mice by the method of Reif and Allen (26). The antiserum was absorbed with AKR brain and thymus cells before use. The cytotoxic titer of the antiserum was measured according to the method of Wigzell (27) with slight modification (28) in terms of ^{51}Cr release from ^{51}Cr-labeled thymus cells in the presence of guinea pig complement; its titer was 640 at 50% target cell lysis.

Rabbit anti-mouse thymocyte serum. (ATS). Antiserum to mouse thymocytes was induced in a rabbit by injection of 2×10^8 A/J murine thymus cells emulsified in complete Freund's adjuvant into the four footpads followed by two intramuscular injections of the same material into the back of animal at 2-week intervals. The serum was collected 10 days after the third injection, heat-inactivated at 56°C for 1 hr and then extensively absorbed with erythrocytes until depleted of detectable hemagglutinins. The serum was further absorbed with A/J bone marrow cells until the lysis of ^{51}Cr-labeled bone marrow cells, measured in terms of release of ^{51}Cr, in the presence of guinea pig complement was reduced to less than 10%.

Complement. Guinea pig fresh serum was used as the source of complement absorbed with agarose (100 mg/ml) for 1 hr at 4°C, according to the method of Cohen and Schelesinger (29), to reduce

its cytotoxicity against mouse thymus cells. At the optimal dilution of the guinea pig serum, *i.e.*, 1:64, it was not cytotoxic itself for mouse thymocytes, but retained sufficient complement activity to lyse all target cells with potent antiserum to these cells.

In vitro treatment of thymus or spleen cells with anti-θ serum or ATS. Washed thymus or spleen cells (4×10^8) were resuspended in 1 ml of the 2-fold diluted anti-θ serum or in 1 ml of 10-fold diluted ATS and incubated for 30 min at 4°C with gentle rotation. After incubation, the cells were washed three times with chilled Hanks' solution by gentle centrifugations, and then resuspended in 1 ml of the 64-fold diluted guinea pig fresh serum (this was the optimal dose of complement) and incubated for 30 min at 37°C. The cells were finally washed with Hanks' solution three times and their viability was established by trypan blue dye exclusion.

Treatment of animals with ATS. To test the *in vivo* effect of ATS on the tumor growth in normal A/J mcie, groups of five normal mice were given several i.v. injections of 0.2 ml of ATS at various times after the tumor cell inoculation. ATS had no cytotoxic activity against Sarcoma 1509a cells in the presence of complement *in vitro*.

Splenectomy. Splenectomy was performed in tumor-bearing mice 7 days after primary s.c. tumor inoculation into the right side of the back. For this purpose, a left lateral incision was made on the back of each tumor-bearing mouse and the abdomen was opened.

The spleen was carefully detached from the pancreas and the splenic vessels were clamped and ligated with silk. The spleen was then removed. The wound was closed with Aron Alpha A (Toa Synthetic Chemical Co. Ltd., Tokyo, Japan).

Results

Detection of immunosuppressor cells in TBA. Inoculation of 10^6 cultured cells of the Sarcoma 1509a into normal A/J mice resulted in the proliferation of tumor cells that finally killed the hosts within 40 days. By contrast, the inoculum of the same number of tumor cells into syngeneic mice rendered highly immune to the tumor was always rejected and disappeared within 2 weeks (Fig. 1).

The existence of immunosuppressor cells in the thymuses or spleens of TBA was substantiated by the effects of these cells on the tumor growth on transfer into immune syngeneic mice at the time of tumor cell inoculation. As is evident from tumor growth curves shown in Figure 1, i.v. adoptive transfer of 4×10^7 washed thymus cells or spleen cells from TBA, which had received a primary tumor inoculum 7 days earlier, into the immune syngeneic mice given subcutaneously 10^6 tumor cells resulted in the suppression of the rejection of the tumors in these animals. On the other hand, adoptive transfer of an equal number of thymus or spleen cells of normal syngeneic mice did not affect the rejection of the tumors (Fig. 1).

As is clearly shown by the tumor growth curves in Figure 2, the thymus cells of TBA suppressed also the ongoing tumor rejection in the immune animals. Thus, transfer of 4×10^7 thymus cells from

TBA 7 days after tumor cell inoculation to the immune syngeneic animals, which had received 10^6 tumor cells 5 days earlier, enhanced the tumor growth significantly, although this effect was temporary. However, normal thymus cells or thymus cells from syngeneic animals bearing an unrelated tumor such as the anaplastic Carcinoma 15091A or lymphoma L1117 had no effect on the rejection of Sarcoma 1509a. These results, therefore, suggested that immunosuppressor cells did exist in the thymuses and spleens of tumor-bearing hosts.

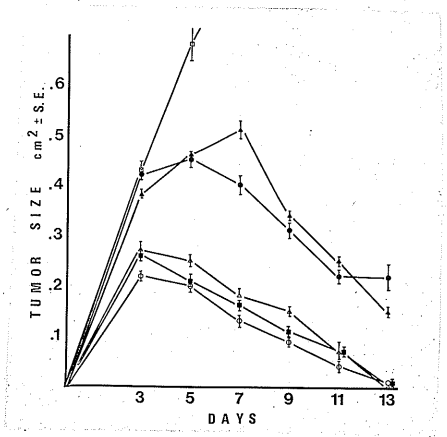

Figure 1. The effect of the thymus or spleen cells of sarcoma 1509a-bearing animals (TBA) on the rejection of the tumor in immune syngeneic animals. Growth curve of the tumor in normal animals receiving 10^6 cultured tumor cells s.c., □——□; rejection curve of the tumor in immune syngeneic animals receiving 10^6 cultured tumor cells s.c., ■——■; suppressive effect of 4×10^7 washed thymus cells (●——●) ($p < 0.001$), or of 4×10^7 spleen cells (▲——▲) ($P < 0.001$) of TBA, transferred i.v. on the rejection of the tumor in immune animals receiving simultaneously 10^6 tumor cells s.c. Control experiment: the effect of 4×10^7 washed normal thymus (○——○), or spleen (△——△) cells, transferred i.v. on the rejection of the tumor in immune animals receiving 10^6 cultured tumor cells. The tumor size is expressed as the mean tumor area ± standard error (S.E.), the tumor area being represented by the product of two perpendicular diameters. Statistical differences between experimental groups and the control group receiving Hanks' solution alone were established by the Student t-test; each group consisted of ten animals.

To confirm these visual findings, the suppressive effect of thymus cells of TBA on the rejection of the tumor in immune syngeneic animals was established by the histologic examination of the tumor

sites of immune animals that had received thymus cells of TBA on days 3,5,7 and 9 after tumor cell inoculation; the control group of mice receive injections of Hanks' solution in lieu of thymus cells of TBA. From the histologic findings given in Figure 3, it may be concluded that mononuclear cell infiltration was inhibited in the tumor tissues of the experimental group that had received thymus cells of TBA, i.e., tumor tissue destruction was limited to and localized only in the center of the tumor, the malignant cells in the peripheral areas of the tumor tissues being still intact at least in day 5. By contrast, the tumor tissues of the control group were totally destroyed by day 5, i.e., marked mononuclear cell infiltration, pyknotic tumor cells, and fragments of nucleus of the tumor cells were observed. Thus, these histologic findings substantiated the macroscopic differences between the tumor size of the experimental group and that of the control group and demonstrated that the immunosuppressor cells of the thymuses or spleens of TBA inhibited mononuclear cell infiltration into the tumor tissues and consequently the destruction of the tumor cells, the net effect manifesting itself as a temporary enhancement of the tumor growth in immune syngeneic animals.

The effect of sera of TBA on the rejection of the tumor in immune syngeneic animals. To examine the effect of sera of TBA on the rejection ofthe tumor, daily i.v. injections of 0.2 ml of the sera, obtained 7 days after tumor cell inoculation, were

given to each immune syngeneic animal for 5 days beginning at the time of tumor cell inoculation. As shown by the data in Table I, these sera had no effect on the tumor rejection in immune animals.

Treatment of thymus or spleen cells of TBA anti-θ or ATS and complement. In order to determine if the suppressive activity was due to T cells, 3×10^8 thymus or spleen cells of TBA were treated with the AKR anti-θ C_3H serum or ATS and guinea pig complement *in vitro*, and then transferred i.v. into syngeneic immune animals that simultaneously received s.c. 10^6 tumor cells into their backs. As is evident from Table II, the treatment with anti-θ serum or ATS and complement did totally abolish the suppressive activity of these cells, thus indicating that immunosuppressor cells belonged to the T cell population.

In vivo effect of ATS on the tumor growth in normal syngeneic animals. To test the effect of ATS on the primary tumor growth in normal syngeneic animals injected with 10^6 Sarcoma 1509a cells s.c. in their backs, ATS was administered to two groups of animals at various times. One group (Gr. I) of five normal animals was given 0.2 ml of ATS i.v. at the time of tumor cell inoculation and 1 day later. The other group (Gr. II) of five normal animals was given 0.2 ml volumes of ATS i.v. on days 3,4,6,8, and 10 after the tumor cell inoculation. Administration of ATS at the time of primary tumor cell inoculation resulted in acceleration of growth of the primary tumor (Gr. I in Fig. 4). On the other hand, when

ATS was given primary tumor cell inoculation, that is, after animals were primed with tumor cells, the growth of primary tumor was clearly suppressed (Gr. II in Fig. 4).

Effect of splenectomy on the growth of tumor in normal syngeneic animals. Since the presence of immunosuppressor T cells in the spleens of TBA was substantiated in the previous experiments, attempts to reduce the immunosuppressor T cell pool in TBA were made by splenectomy 7 days after the tumor cell inoculation. (continued page 83)

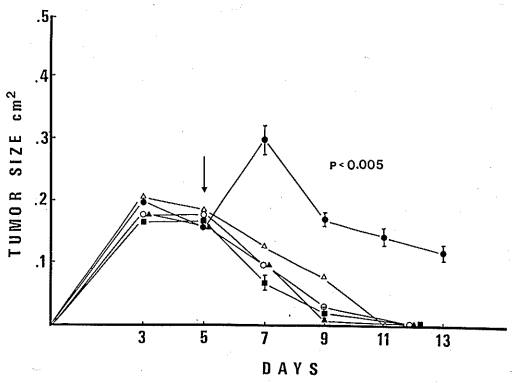

Figure 2. The suppressive effect of thymus cells of TBA on the ongoing tumor rejection in immune syngeneic animals. Washed thymus cells (4×10^7) obtained from A/Jax mice bearing the relevant tumor sarcoma 1509a (●——●), or unrelated tumors: anaplastic Carcinoma (O——O) and Lymphoma L1117 (Δ——Δ), or from normal syngeneic animals (▲——▲), were transferred to each immune syngeneic animal of each group, respectively, which had received 10^6 1509a cells 5 days earlier. In an additional control group, immune animals received only an injection of Hanks' solution (■——■). Each group contained 5 immune syngeneic animals. The tumor size was expressed in the same manner as in Figure 1. Statistical differences between experimental groups and the control group receiving Hanks' solution was calculated by the Student t-test.

TABLE I

The effect of the sera of TBA on the rejection of the tumor in immune syngeneic animals

Group No.[a]	Treatment[b]	Tumor Size[c] in cm²			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area[d] ± S.E.			
I	TBA serum	0.26 ± 0.01 (NS)	0.33 ± 0.02 (NS)	0.26 ± 0.01 (NS)	0.16 ± 0.01 (NS)
II	Normal serum	0.26 ± 0.01 (NS)	0.31 ± 0.01 (NS)	0.20 ± 0.01 (NS)	0.15 ± 0.01 (NS)
III	Hanks' solution	0.28 ± 0.01	0.28 ± 0.01	0.22 ± 0.01	0.14 ± 0.01

[a] Each group consisted of ten immune syngeneic mice.

[b] Immune syngeneic animals were treated by daily i.v. injections of 0.2 ml of serum of TBA, normal serum or Hanks' solution for 5 consecutive days beginning at the time of tumor cell (1×10^6) inoculation.

[c] Sarcoma 1509a (1×10^6 viable cells in 0.1 ml) was inoculated s.c. into the backs of immune syngeneic animals.

[d] Tumor areas were considered to be approximately equal to the product of two perpendicular diameters of the tumor and the results are expressed in cm², as the mean of the area ± standard error of the mean (S.E.). Statistical differences between experimental groups and the control group (III) were calculated by the Student t-test and were expressed by p values.

[e] NS, statistically not significant.

TABLE II

Effect of treatment of thymus or spleen cells of TBA with anti-θ serum or ATS and complement in vitro on the tumor rejection

Group No.[a]	Treatment[b]	Tumor Size[c] in cm²			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± SE			
I	Thymus cells of TBA non-treated	0.40 ± 0.02 ($p < 0.005$)	0.58 ± 0.02 ($p < 0.001$)	0.50 ± 0.01 ($p < 0.001$)	0.40 ± 0.02 ($p < 0.001$)
II	Thymus cells of TBA treated with anti-θ and C[d]	0.27 ± 0.01 (NS)[e]	0.28 ± 0.01 (NS)	0.21 ± 0.02 (NS)	0.15 ± 0.01 (NS)
III	Thymus cells of TBA treated with ATS and C[d]	0.30 ± 0.01 (NS)	0.33 ± 0.02 (NS)	0.25 ± 0.01 (NS)	0.20 ± 0.01 (NS)
IV	Spleen cells of TBA non-treated	0.45 ± 0.02 ($p < 0.001$)	0.49 ± 0.01 ($p < 0.001$)	0.48 ± 0.02 ($p < 0.001$)	0.37 ± 0.01 ($p < 0.001$)
V	Spleen cells of TBA treated with anti-θ and C[d]	0.28 ± 0.01 (NS)	0.28 ± 0.02 (NS)	0.23 ± 0.01 (NS)	0.17 ± 0.01 (NS)
VI	Spleen cells of TBA treated with ATS and C[d]	0.29 ± 0.01 (NS)	0.29 ± 0.01 (NS)	0.21 ± 0.01 (NS)	0.16 ± 0.01 (NS)
VII	Control Hanks' solution	0.30 ± 0.02	0.32 ± 0.01	0.21 ± 0.01	0.21 ± 0.01

[a] Each group consisted of ten immune syngeneic mice.

[b] Immune syngeneic mice received i.v. either 3×10^7 non-treated lymphoid cells or 3×10^7 eq treated cells with anti-θ serum or ATS and guinea pig complement (C) *in vitro* at the time of tumor cell inoculum. In the control group, animals were given i.v. Hanks' solution alone.

[c] The tumor size was calculated as indicated in footnote *d* of Table I.

[d] Thymus or spleen cells obtained from TBA were evenly divided into three parts (3×10^8/each part). One part was untreated and the other two parts were treated with either anti-θ or ATS and guinea pig complement (C) according to the procedure described in *Materials and Methods*. After treatment, cells were washed with Hanks' solution and resuspended in the initial volume of Hanks' solution; viable cells were counted. Anti-θ serum eliminated 95% of thymus cells and 48% of spleen cells of TBA; ATS eliminated 99% of thymus cells and 50% of spleen cells in the presence of guinea pig complement.

[e] NS, statistically not significant.

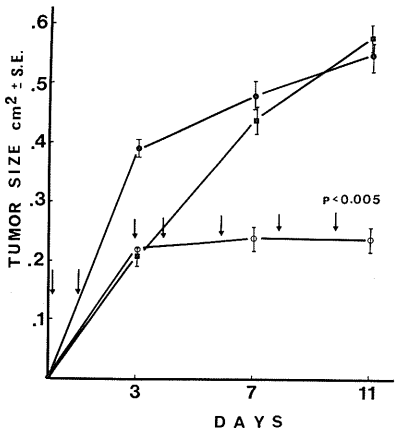

Figure 4. The effect of ATS on the growth of the tumor in normal syngeneic animals. Five mice of group I (●) were treated by injections of 0.2 ml of ATS at the time of tumor cell (1×10^6) inoculation (day 0) and on the following day (day 1). Five mice of group II (○) were treated by injections of 0.2 ml of ATS at day 3, 4, 6, 8, and 10 after tumor cell (1×10^6) inoculation. Five mice of Group III (■) were non-treated control. The statistical significance between Group II and the control (Group III) was calculated by the Student t-test.

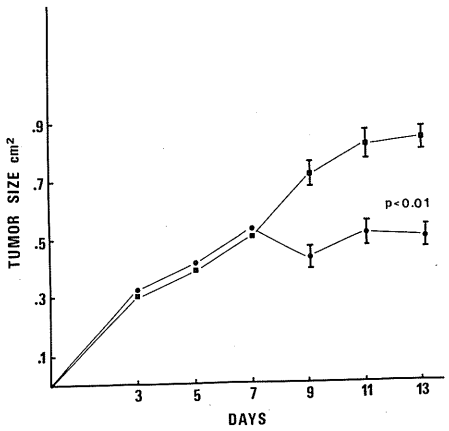

Figure 5. The effect of splenectomy on the growth of the tumor in normal syngeneic animals. Ten animals of Group I (●) were splenectomized at day 7 after tumor cell (1×10^6) inoculation. The ten animals of Group II (■) were used as controls. The results are expressed by means of duplicate experiments.

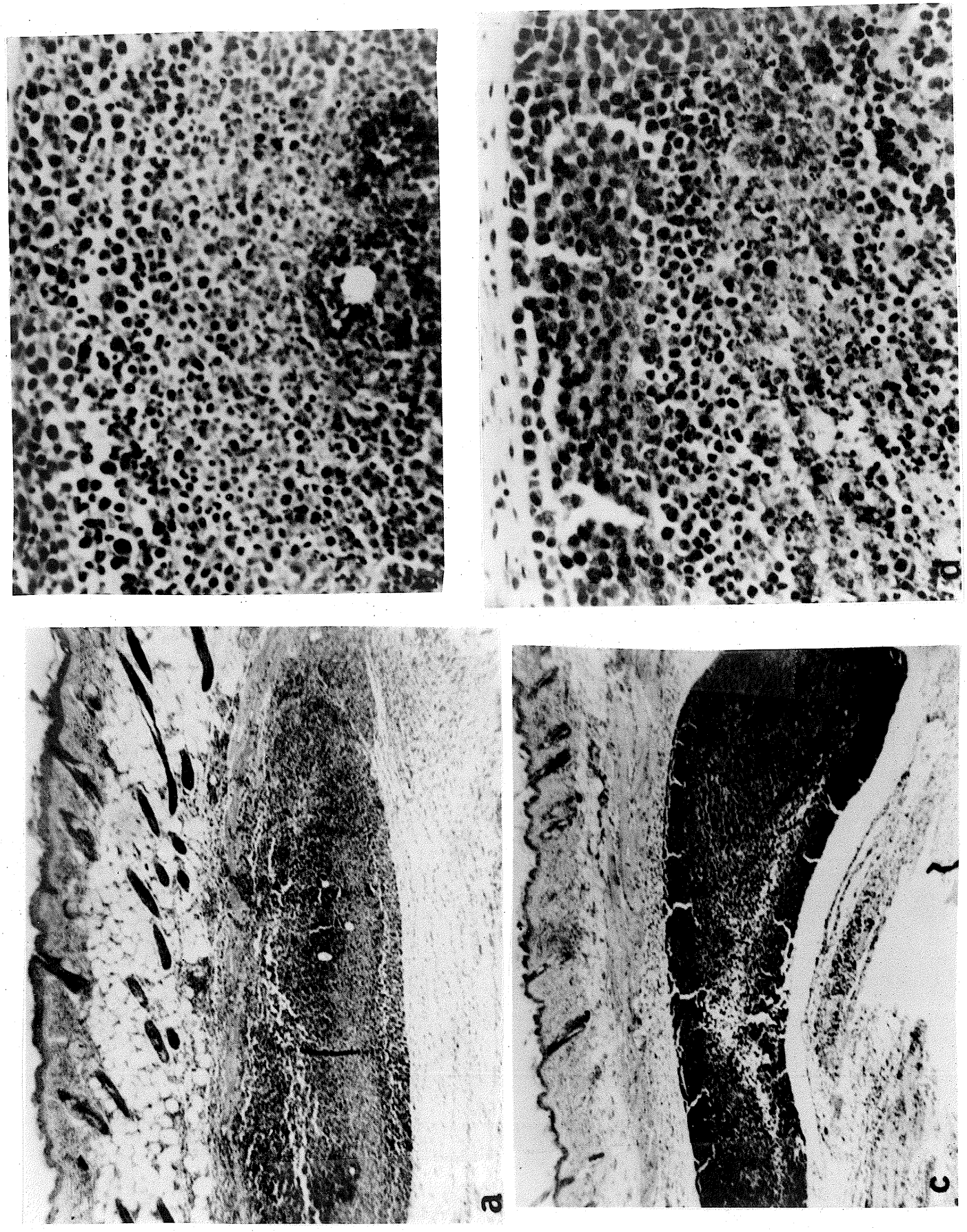

FIGURE 3

FIGURE 3

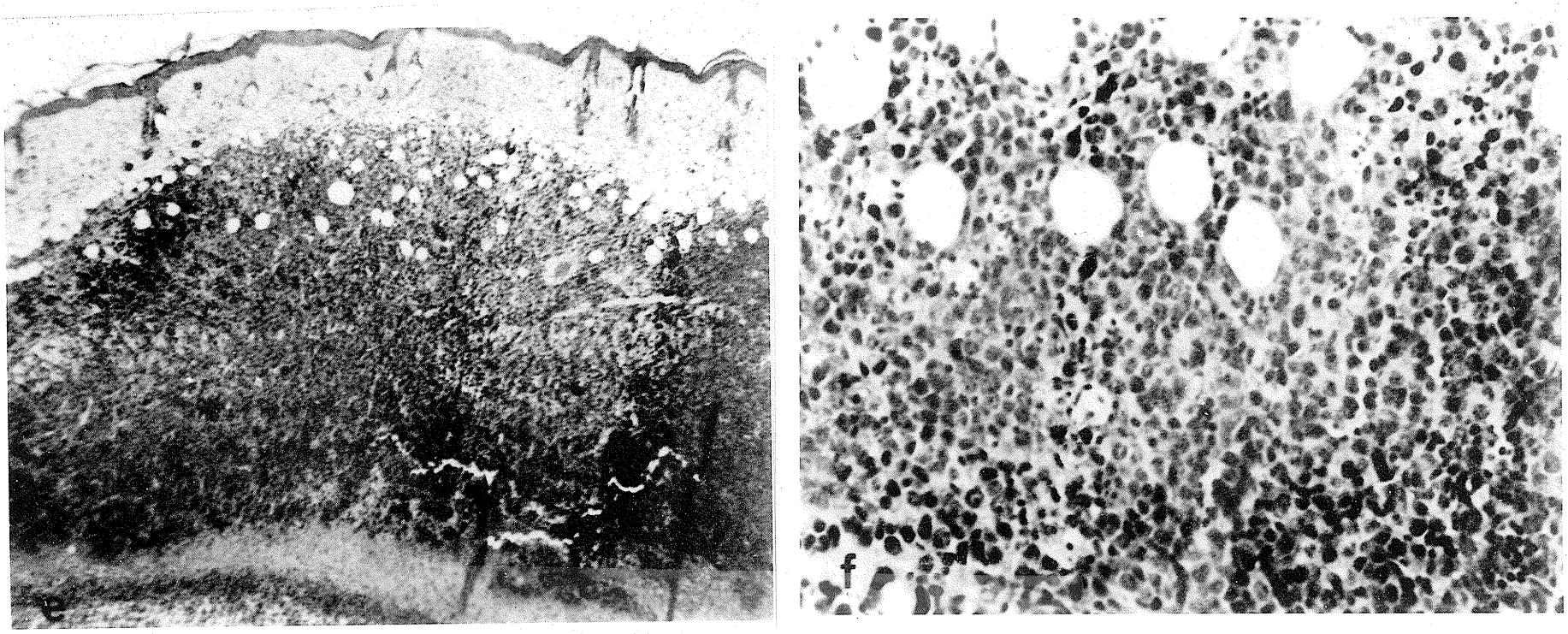

Figure 3. Histologic findings. *a*, Tumor tissue section of control group at day 5 after tumor cell inoculation (1×10^6) into immune syngeneic animals, stained with hematoxylin and eosin ($\times$ 40). There is marked mononuclear cell infiltration in the whole area of the tumor tissue, i.e., the tumor tissue is totally destroyed. *b*, Higher magnification ($\times$ 160) of *a*. Tumor tissue is mostly necrotic and any tumor cells remaining in the marginal region are pyknotic. *c*, Tumor tissue section of experimental group at day 5 which had received 4×10^7 thymus cells of TBA at the time of tumor cell inoculation ($\times$ 40). The mononuclear cell infiltration is clearly inhibited and necrosis of tumor tissue is limited to the core of the tumor. *d*, Higher magnification ($\times$ 160) of *c*. Tumor cells in the marginal region of the tumor remain intact. *e*, Additional control: tumor tissue section at 5 days after inoculation of 1×10^6 tumor cells into group of normal syngeneic animals ($\times$ 40). Infiltration of tumor cells into normal tissue and their proliferation. *f*, Higher magnification ($\times$ 160) of *e*. Massive infiltration of tumor cells; some tumor cells appear to be in mitosis.

As demonstrated by the data shown in Figure 5, splenectomy resulted in significant suppression of the primary tumor growth in normal syngeneic animals, whereas sham splenectomy (data not shown) had no effect.

DISCUSSION

The results obtained in this study clearly indicate that "immunosuppressor" cells capable of inhibiting the rejection of the tumor in immune syngeneic animals are present in the thymuses and spleens of TBA. Although the net suppression of the immune response to the tumor by transfer of thymus or spleen cells of TBA was temporary, these findings demonstrate unequivocally that immunosuppression was achieved in highly immune animals and consequently it may be concluded that the lymphoid cells of TBA were highly immunosuppressive. Hence, one may infer that normally a weak immune response may be mounted by primary hosts and that it may be overshadowed by the negative suppressive effect of immunosuppressor cells in TBA.

Since thymus or spleen cells of normal syngeneic animals had no immunosuppressive activity, it is suggested that the inhibiting cells are generated in the syngeneic hosts by tumor cell inoculation.

Furthermore, from the evidence that T lymphocytes are unlikely to return to the thymus after the cells emigrate into circulation (30), these results also indicate that immunosuppressor cells in the thymus are generated primarily *in situ*. Several investigations have already identified antigen-binding cells in the thymus or antigen-primed animals (31-33).

Moreover, recently Okumura and Tada (17) have found inhibitory T cells, capable of specifically regulating IgE antibody formation to dinitrophenyl (DNP), in the thymuses of rats immunized with DNP-Ascaris, which would suggest that some antigens may get to the thymus. Hence, by analogy, it is not unreasonable to assume that soluble tumor antigens shed by malignant cells might also reach the thymus and stimulate appropriate immunocompetent cells.

The supppressive effect of thymus and spleen cells of TBA on the rejection of the tumor appears to be specific for the corresponding tumor cells. The specificity of the immunosuppressor cells in the tumor-bearing hosts has been demonstrated by us in more detail in a separate series of experiments (manuscript in preparation). The immunosuppressive effect of thymus or spleen cells of TBA was abrogated by treatment with anti-θ antiserum or ATS and complement, thus indicating that the observed immunosuppression was mediated by T lymphocytes. It is well recognized that rejection of tumor in immune animals is due to its destruction mainly by T effector lymphocytes (effector T cells) (4,5). Therefore, these results suggest that the immunosuppressor T cells might interfere directly with the manifestation of the effector T cells against the tumor cells. However, since there is increasing evidence that cell-to-cell cooperation ($T_1 \leftrightarrow T_2$ cell cooperation) does actually exist in cellular manifestations of the immune response, e.g., in the GVH response (13,14) or lymphocyte-mediated target cell lysis *in vitro*

(16), the problem of the exact site of action of "immunosuppressor" T cells on the suppression of tumor rejection remains to be elucidated. Since formation of antibodies against the tumor in syngeneic animals has not been examined in this study, it is impossible to state if the immunosuppressor T cells might also affect the formation of antibodies to the tumor in syngeneic animals.

The treatment of normal syngeneic animals with ATS had two distinct effects on the growth of the tumor. While administration of ATS to normal animals at the time of tumor cell inoculation caused acceleration of the growth of the tumor, ATS given after tumor cell inoculation resulted in suppression of the growth of the tumor. These findings, therefore, suggest that effector cells as well as immunosuppressor T cells are activated in the hosts by tumor cell inoculation. However, administration of ATS eliminates or inactivates the whole T cell population if given before priming the immunocompetent cells of the hosts with tumor cells. Thus, the total immune response is suppressed and the growth of the tumor is enhanced. On the other hand, after priming the different types of immunocompetent cells, if immunosuppressor T cells were more sensitive to ATS than the other primed T cell populations, treatment with ATS at this stage would inactivate selectively the immunosuppressor T cells, resulting in a net suppression of tumor growth. However, the alternate exaplanation that ATS might activate or stimulate effector

T cells, once the immunocompetent cells of the hosts are primed with tumor cells, cannot be ruled out; the net effect of this phenomenon would also be an inhibition of tumor growth.

The effects of ATS on the cell-mediated as well as on the humoral immune response have been investigated by many workers (34-40). Thus, in relation to immunity to transplantation of allogeneic cells, which is thought to be mainly a cell-mediated response, ATS has a strong immunosuppressive effect on the rejection of allografts when it is given shortly before the transplantation, or at the time of the transplantation. However, ATS has little immunosuppressive effect on an ongoing allograft rejection. Moreover, it has been recently reported by several investigators that ATS enhanced antibody formation to some antigens such as Type III pneumococcal polysaccharide (20,39,40) and it has been suggested that suppressor T cells might be more sensitive to ATS treatment than other immunocompetent cells *in vivo* after priming. Although in the present study, the observations were limited to cell-mediated immunity, one may suggest that the effect of ATS on antibody formation appears to be paralleled by our findings. However, since the exact mechanism of the effect of ATS on the immune response has not been clarified, the site of action of ATS on immune responses mediated by T cells remains to be established. Splenectomy in TBA has been shown to lead to increased resistance in mice to the transplant of the syngeneic methylcholanthrene-induced sarcoma (41);

this effect has been interpreted as being due to a decreased production of blocking antibody on splenectomy. In essence, a similar effect was observed in the present system. However, the present experimental results showed that washed spleen cells of TBA had a suppressive effect on the rejection of the tumor and that this effect was entirely abolished by treatment of these spleen cells with anti-θ serum and complement. Moreover, the sera of TBA had no suppressive or blocking effect on the rejection of the tumor in immune syngeneic animals, thus suggesting that splenectomy would most likely cause the removal of the main pool of immunosuppressor T cells.

Attempts to establish if B cells were able to inhibit the rejection of the tumor in immune syngeneic animals were unsuccessful. Consequently, one may postulate that the existence of immunosuppressor T cells in TBA may be a major contributing feature of tumor-host relationships favoring the growth of an antigenic tumor and resulting in an apparent lack of an effective immune response.

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CHAPTER 2

THE NATURE OF IMMUNOSUPPRESSOR T CELLS IN TUMOR-BEARING HOSTS

In the preceding Chapter , tumor-bearing animals (TBA) were shown to possess immunosuppressor cells endowed with the capacity of regulating negatively the immune response to the methylcholanthrene-induced tumor Sarcoma 1509a. Thus, suppression of tumor rejection in syngeneic animals immune to this tumor was accomplished by the transfer of thymus or spleen cells of TBA; by contrast, adoptive transfer of thymus or spleen cells of normal syngeneic animals into immune syngeneic animals did not affect the growth of the tumor. Moreover, the treatment of thymus or spleen cells of TBA with anti-θ, serum or anti-mouse thymocyte serum (ATS) and complement entirely abolished the suppressive effect of these cells. It was, therefore, concluded that the immunosuppressor cells belonged to the T cell population and were activated in the thymuses or spleens of the hosts by the tumor cell inoculum.

The present study was undertaken with a view to establishing the characteristics of these immunosuppressor cells present in TBA.

Materials and Methods

Animals. Three- to 5-month-old A/Jax mice, purchased from the Jackson Laboratory, Bar Harbor, Maine, were used throughout the experiments.

Tumor cells. Methylcholanthrene-induced Sarcoma 1509a cells, transplantable in A/Jax mice were isolated as described earlier (1). The 1509a cells obtained from ascites of the TBA were cultured in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.)

supplemented with 5% fetal calf serum (FCS). The tumor cells were used in all experiments after at least three *in vitro* subcultures.

Tumor inoculation and excision. Cultured tumor cells were washed with cold Hanks' solution three times and 10^6 cells in 0.1 ml of Hanks' solution, were subcutaneously injected into the center of the back of each animal after removal of its hair by clippers.

Seven days later, the tumor tissue was carefully cut out together with adherent normal skin by scissors and the wound was closed with chemical adhesive Aron Alpha (Toa Synthetic Chemical Co., Ltd., Tokyo, Japan).

Thymus, spleen and lymph node cells of TBA. Thymuses, spleens and lymph nodes were extirpated from TBA 7 days after inoculation of tumor cells. The thymuses were carefully separated from perithymic lymph nodes and removed by fine forceps. The cervical, axillary and inguinal lymph nodes served as the source of lymph node cells. The thymuses, spleens and lymph nodes were gently pressed and ground in a coarse glass grinder with chilled Hanks' solution separately. The suspension of the cells of each organ in 20 ml of chilled Hanks' solution was passed through a 200-mesh stainless steel screen. Cells were washed four times with 20 ml of chilled Hanks' solution and finally resuspended in 2.0 ml of Hanks' solution. Nucleated viable cells were counted in the hemocytometer by trypan blue dye exclusion.

Bone marrow cells. Bone marrow cells were obtained from the femora of TBA by flushing out the marrow cavity with chilled Hanks' solution. Cells were washed four times with Hanks solution and

counted as described above.

Peripheral blood lymphocytes. Peripheral lymphocytes were collected from the pool of blood obtained from 50 heparinized TBA by cutting the right axillary artery after opening the skin. The pool of blood was mixed with 1/5 volume of 6% dextran (m.w. 110,000, Pharmacia Uppsala, Sweden) in plastic syringes and allowed to stand for 1 hr at room temperature. The white cell layer was collected and pooled in a plastic culture flask (250 ml Culture Flask, Falcon, Oxnard, Calif.) To remove adherent cells, the white cells were maintained in the culture flask for 90 min. at 37°C in a CO_2 incubator. After incubation, nonadherent cells were collected and washed with Hanks' solution four times.

Treatment of bone marrow cells *in vitro* with AKR anti-θ C_3H serum. The procedure was described in the preceding article (1). Briefly, washed bone marrow cells were resuspended in 1 ml of the 2-fold diluted AKR anti-θ C_3H serum for 30 min at 4°C with gentle rotation. The cells were collected by gentle centrifugation, washed with chilled Hanks' solution, and resuspended in 1 ml of the 64-fold diluted guinea pig fresh serum (this was the optimal dose of complement) and incubated for 30 min at 37°C. The cells were then washed with chilled Hanks' solution three times and the viable nucleated cells were counted by trypan blue dye exclusion.

Hydrocortisone treatment. To obtain cortisone-resistant thymus or spleen cells from TBA, each mouse was given i.p. 2.5

mg of hydrocortisone (Upjohn, Kalamazoo, Mich.) 5 days after tumor cell inoculation. Two days after hydrocortisone treatment of TBA, thymuses or spleens served for the preparation of the appropriate cell suspensions.

Discontinuous Ficoll density gradient centrifugation for cell separation. The spleen cells were separated by centrifugation through discontinuous density gradient of Ficoll (Pharmacia Uppsala, Sweden). Twenty, 25, and 30% (w/v) of Ficoll solutions were prepared in RPMI 1640 medium supplemented with 10% FCS at 4°C. Seven milliliters of each of these three Ficoll solutions were layered in a 30-ml cellulose nitrate tube (Beckman Instruments, Inc., Fullerton, Calif.) Spleen cells of TBA were applied on the top layer and centrifuged at 9400 x G for 30 min with a SW-27 rotor. After centrifugation, the cells present in each layer were collected separately and washed with chilled Hanks' solution three times. The nucleated viable cells were counted by trypan blue dye exclusion.

In vivo assay of immunosuppressor (IS) cells. To examine the immunosuppressive effect of prepared lymphoid cells, an *in vivo* assay system developed in the earlier study (1) was used. Briefly, lymphoid cells obtained from Sarcoma 1509a-bearing animals were transferred i.v. into immune syngeneic animals at the time of the tumor inoculation. The effect of prepared lymphoid cells from TBA on the rejection of the tumor in immune syngeneic animals was examined by measuring the perpendicular diameters of the tumor.

The tumor size was expressed as the mean tumor area ± standard error of the mean (S.E.), the tumor area being represented by the product of two perpendicular diameters. The tumor size was expressed as the mean tumor area ± standard error of the mean (S.E.), the tumor area being represented by the product of two perpendicular diameters.

RESULTS

Appearance of IS cells in hosts after tumor inoculation. In the previous study, it had been noted that IS cells were generated in the thymuses of the hosts after inoculation of Sarcoma 1509a cells. In order to investigate the time of appearance of the IS cells, thymus cells were obtained from five experimental groups of animals (each consisting of six mice), 1,3,5,7, or 16 days after inoculation of 1×10^6 tumor cells into each mouse, the suppressive effect of the thymus cells of each of these groups on the rejection of the tumor in syngeneic immune animals was then tested. The washed thymus cells (4×10^7) of each group were transferred i.v. into immune animals at the time of tumor cell reinoculation (1×10^6). It is obvious from the data listed in Table I that within 1 day after inoculation of tumor cells into non-immune animals, the host's thymus cells possessed suppressive activity.

Disappearance of IS cells in hosts after tumor excision. The kinetics of IS cells in hosts after tumor extirpation was also examined. In this experiment, all mice received an inoculum of 1×10^6 tumor cells 7 days before tumor excision. The thymus cells were obtained from three experimental groups of animals (each

consisting of eight mice), from which the tumor had been removed 1, 5, and 9 days before and their suppressive effect on the rejection of the tumor in syngeneic immune animals was tested, as described earlier.

As is evident from the data in Table II, the thymus cells did not confer any immunosuppressive activity even 5 days after tumor extirpation; these findings suggest that IS cells are short-lived once the tumor is removed.

Dose effect of IS cells on the suppression of the tumor rejection in immune syngeneic animals. To examine the minimum effective dose of IS cells for the suppression of the tumor rejection, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 thymus cells of TBA were given i.v. to each group of five immune animals, respectively, at the time of tumor cell inoculation (1×10^6). As is evident from Table III, suppression of tumor rejection was observed in the groups that had received more than 1×10^7 thymus cells and that the degree of suppression was dependent on the dose of thymus cells of TBA.

Distribution of IS cells in TBA. In order to investigate the distribution of the IS cells in lymphoid organs of TBA, lymphocytes were obtained from circulating blood, draining lymph nodes, bone marrow, spleens, and thymuses of TBA 7 days after tumor cell inoculation, and tested separately for their suppressive effect on tumor rejection. Forty million lymphocytes from each source were administered per immune syngeneic animal of each group at the time

of tumor cell inoculation. The data listed in Table IV shows that bone marrow and draining lymph node cells, as well as thymus and spleen cells, had similar suppressive effects, whereas blood lymphocytes did not show any significant suppressive activity. These results are interpreted as indicating that IS cells might not be present in circulation in large numbers.

IS Cells in bone marrow of TBA. Since bone marrow cells of TBA had a similar suppressive effect as spleen or thymus cells on tumor rejection, bone marrow cells were subjected to treatment with anti-θ serum and complemented to establish if their immunosuppressive activity was also due to T cells. From the data shown in Table V, it can be concluded that although this treatment eliminated not more than 6% of bone marrow cells, it completely abolished their suppressive activity, thus suggesting that the immunosuppressive activity is associated with T cells in the bone marrow.

The suppressive effect of cortisone-resistant thymus and spleen cells. In order to test whether or not the IS cells were cortisone-resistant, thymus or spleen cells were collected from TBA 2 days after i.p. administration of 2.5 mg of hydrocortisone per mouse; these mice had received the hydrocortisone 5 days after tumor cell inoculation. The washed hydrocortisone-resistant thymus or spleen cells were transferred i.v. into immune syngeneic animals at the time of tumor cell reinoculation; in the control experiment, a larger dose of thymus and spleen cells of TBA was

injected i.v. into immune animals together with the tumor cells. As shown in Table VI, hydrocortisone-resistant thymus or spleen cells significantly suppressed the tumor rejection in immune animals and their suppressive activity was equivalent to that of a larger dose of thymus or spleen cells from TBA that had not received the hydrocortisone treatment. It may be inferred, therefore, that IS cells are cortisone-resistant and that *in vivo* hydrocortisone treatment results in concentration of activated IS cells in the thymuses of spleens of TBA.

Distribution of IS cells in spleen cell fractions of TBA, isolated density gradient centrifugation. After centrifugation of IS cells through the Ficoll density gradient, samples of 5×10^6 cells were collected from the four layers and transferred i.v. into four groups of five immune syngeneic animals at the time of tumor cell inoculation. It is obvious from the data given in Table VII that the cells isolated in the top layer (20% Ficoll layer) had significant suppressive effect, whereas the cells in the heavier region of the gradient had no suppressive effect. It may, therefore, be concluded that IS cells belong to a light cell population.

DISCUSSION

From the results of this study, we may derive a number of characteristics of IS cells of TBA which are summarized below:

1) IS cells in syngeneic hosts are generated by tumor cell inoculation within 24 hr and seem to persist as long as the tumor

grows in the hosts; however, IS cells disappeared in the hosts within 5 days after removal of the tumor. 2) The suppressive effect on tumor rejection in immune syngeneic animals was dependent on the dose of thymus cells of TBA; a dose higher than 10×10^6 thymus cells showed significant suppression. 3) The IS cells were demonstrated to be present in the thymus, spleen, bone marrow, and draining lymph nodes of TBA, but their activity could not be demonstrated in the blood of TBA. Immunosuppressive activity which was associated with bone marrow cells was also totally abolished by treatment with anti-θ serum and complement. 4) Treatment of TBA with hydrocortisone did not abolish the suppressive activity of thymus and spleen cells thus indicating IS cells were cortisone-resistant. 5) The suppressive activity was associated with cells present in the lowest density layer separated by Ficoll density gradient centrifugation; it is therefore concluded that IS cells are a light cell population.

IS cells could be stimulated by tumor cell inoculation in the lymphoid organs of the hosts even within 24 hr. It is not unreasonable to consider that the generation of IS cells might be triggered by contact between IS cells and soluble tumor antigens which appeared to be released from the inoculated tumors and that these antigens reached the thymus, spleen, and other lymphoid organs of TBA. In this connection, it might be pointed out that there is increasing evidence that soluble tumor antigens are found in sera of tumor-bearing hosts (2-5). In non-tumor systems, it has been

reported that suppressor T cells can be generated in the hosts by diverse stimuli of highly immunogenic heterologous proteins, e.g., DNP-Ascaris (6), bovine γ-globulin (7), sheep erythrocytes (8), Keyhole limpet hemocyanin, (9), and pneumococcal polysaccharide SIII, (10). Interestingly, in this study, the immunosuppressive activity could be detected in the thymuses of TBA within 24 hr after tumor cell inoculation in spite of the apparently weak immunogenicity of tumor antigens. At the present time, the mechanism underlying the generation of immunosuppressor cells or the induction of negative cellular interactions still remains to be elucidated. Thus, no consistent picture has yet emerged for the appearance of suppressor T cells in hosts during immunization. In the tumor system described, the activation of IS cells might represent an earlier immunologic event than antibody production in the tumor hosts, although there is no firm data to support this idea. Furthermore, whereas IS cells seem to persist in TBA as long as the tumor is progressing, these cells disappear within 5 days after removal of the tumor and the mice acquire immunity to the tumor; these findings suggest that IS cells are only active in the hosts in the presence of tumor.

At first sight, on the basis of the evidence presented, one might be inclined to suggest that the observed immunosuppression might be caused by tumor antigens bound to the receptors of immunocompetent cells in the hosts and in particular to T cells, since the *in vitro* treatment of thymus or spleen cells with anti-θ serum and complement abolished their immunosuppressive activity. However, this possibility

can be ruled out on the basis of recent findings (11) that soluble factor(s) with identical immunosuppressive capacity was isolated from the extracts of IS cells, obtained by freezing and thawing, and that this factor(s) was absorbed with the homologous Sarcoma 1509a cells.

IS cells are localized, not only in the thymuses and spleens, but also in draining lymph nodes and even in the bone marrow of TBA. The IS cells in the bone marrow of TBA were also effective in suppressing the tumor rejection in immune syngeneic animals. Since the *in vitro* treatment of bone marrow cells of TBA with anti-θ serum and complement which lysed only 6% of the bone marrow cells completely abolished their suppressive effect, this immunosuppression also appeared to be mediated by T cell population in bone marrow. Nevertheless, it must be pointed out that normally there are fewer T cells among bone marrow cells and, therefore, one may suggest that tumor antigens or some factor elaborated by tumor cells might generate more IS cells in bone marrow than other antigens. In any case, the data obtained in this study are supporting the recent report of the Herzenbergs and their colleagues (12) that suppressor T cells are found even in the bone marrow of mice whose allotypes have been suppressed. As in the studies of Baker *et al.* (13), IS cells were not detected in this study among the circulating blood lymphocytes. Thus, one may suggest that IS cells do not seem to have circulating functions. Moreover, hydrocortisone treatment of TBA resulted in the concentration of IS cells in the thymuses and spleens, thus

TABLE I

Appearance of IS cells in hosts after tumor cell inoculum

Group No.[a]	Treatment[b]	Tumor Size[c] in cm^2			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± S.E.			
I	Thymus cells of NA[d]	0.29 ± 0.02 (NS)[e]	0.23 ± 0.01 (NS)	0.16 ± 0.01 (NS)	0.14 ± 0.01 (NS)
II	Thymus cells of TBA[f] 1 day post-tumor inoculum	0.44 ± 0.02 ($p < 0.005$)	0.47 ± 0.02 ($p < 0.001$)	0.34 ± 0.01 ($p < 0.001$)	0.30 ± 0.01 ($p < 0.001$)
III	Thymus cells of TBA 3 days post-tumor inoculum	0.49 ± 0.02 ($p < 0.001$)	0.41 ± 0.01 ($p < 0.001$)	0.32 ± 0.01 ($p < 0.001$)	0.26 ± 0.01 ($p < 0.001$)
IV	Thymus cells of TBA 5 days post-tumor inoculum	0.50 ± 0.01 ($p < 0.001$)	0.41 ± 0.01 ($p < 0.001$)	0.32 ± 0.004 ($p < 0.001$)	0.26 ± 0.01 ($p < 0.001$)
V	Thymus cells of TBA 7 days post-tumor inoculum	0.46 ± 0.01 ($p < 0.001$)	0.39 ± 0.01 ($p < 0.001$)	0.32 ± 0.004 ($p < 0.001$)	0.26 ± 0.01 ($p < 0.001$)
VI	Thymus cells of TBA 16 days after post-tumor inoculum	0.47 ± 0.01 ($p < 0.001$)	0.47 ± 0.01 ($p < 0.001$)	0.35 ± 0.003 ($p < 0.001$)	0.24 ± 0.004 ($p < 0.001$)
VII	Control Hanks' solution	0.30 ± 0.01	0.26 ± 0.01	0.19 ± 0.004	0.12 ± 0.01

[a] Each experimental group contained 5 immune syngeneic animals and control group (VII) contained 10 immune syngeneic animals. All immune animals were given s.c. 1×10^6 tumor cells.

[b] Thymus cells were obtained from TBA of each group of mice which had received 10^6 tumor cells 1, 3, 5, 7, and 16 days before, respectively; the cells (5×10^7) were transferred into each immune syngeneic animal at the time of tumor challenge (1×10^6). As controls, 5×10^7 thymus cells of normal or Hanks' solution were given to immune syngeneic mice.

[c] Tumor size was expressed as tumor area calculated by multiplication of two perpendicular diameters of the tumor. The results are shown as the mean tumor area of five animals in each group ± standard error of the mean (S.E.).

[d] Normal animals.

[e] NS, not statistically significant.

[f] Tumor-bearing animals.

TABLE II

Disappearance of IS cells in hosts after tumor excision

Group No.[a]	Treatment[b]	Tumor Size in cm^2			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± SE			
I	Thymus cells of TBA[c]	0.46 ± 0.01 ($p < 0.001$)	0.39 ± 0.01 ($p < 0.001$)	0.32 ± 0.004 ($p < 0.001$)	0.27 ± 0.003 ($p < 0.001$)
II	Thymus cells from hosts 1 day after tumor removal	0.43 ± 0.01 ($p < 0.001$)	0.40 ± 0.01 ($p < 0.001$)	0.38 ± 0.01 ($p < 0.001$)	0.22 ± 0.01 ($p < 0.001$)
III	Thymus cells from hosts 5 days after tumor removal	0.35 ± 0.02 ($p > 0.01$)	0.30 ± 0.01 ($p > 0.01$)	0.20 ± 0.01 (NS)[d]	0.11 ± 0.01 (NS)
IV	Thymus cells from hosts 9 days after tumor removal	0.29 ± 0.01 (NS)	0.25 ± 0.01 (NS)	0.18 ± 0.004 (NS)	0.08 ± 0.003 (NS)
V	Control Hanks' solution	0.30 ± 0.01	0.25 ± 0.01	0.19 ± 0.004	0.12 ± 0.01

[a] Each experimental group consisted of five immune syngeneic animals. Control group (V) contained ten immune syngeneic mice. Each immune animal was given s.c. 1×10^6 tumor cells.

[b] Thymus cells were obtained separately from three experimental groups of animals (each consisting of eight mice) from which tumor had been removed 1 day, 5 days, and 9 days before, respectively. The animals of each group had been inoculated with 1×10^6 tumor cells 7 days before removal of the tumor. Fifty million thymus cells from each group were then transferred into immune syngeneic animals of each group, respectively, at the time of tumor cell challenge (1×10^6). In control groups, Hanks' solution or 5×10^7 thymus cells of TBA which had received 1×10^6 tumor cells 7 days earlier, were given to immune syngeneic animals. Statistical differences between experimental groups and control group with Hanks' solution alone were established by the Student t-test and expressed by p value.

[c] Tumor-bearing animals.

[d] NS, not statistically significant.

TABLE III

Dose effect of IS cells on the suppression of the tumor rejection

Group No.[a]	Dose of Thymus Cells of TBA[b]	Tumor Size in cm²			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± S.E.			
I	1×10^8 cells	0.56 ± 0.01 ($p < 0.001$)	0.54 ± 0.01 ($p < 0.001$)	0.43 ± 0.01 ($p < 0.001$)	0.39 ± 0.02 ($p < 0.001$)
II	1×10^7 cells	0.46 ± 0.01 ($p < 0.01$)	0.44 ± 0.01 ($p < 0.001$)	0.30 ± 0.02 ($p < 0.05$)	0.29 ± 0.01 ($p < 0.001$)
III	1×10^6 cells	0.36 ± .01 ($p < 0.05$)	0.30 ± 0.01 ($p < 0.05$)	0.24 ± 0.01 ($p < 0.02$)	0.17 ± 0.01 ($p < 0.1$)
IV	1×10^5 cells	0.29 ± 0.01 (NS)	0.27 ± 0.01 (NS)	0.19 ± 0.01 (NS)	0.17 ± 0.01 (NS)
V	1×10^4 cells	0.26 ± 0.01 (NS)	0.23 ± 0.01 (NS)	0.18 ± 0.01 (NS)	0.14 ± 0.01 (NS)
VI	Control Hanks' solution	0.29 ± 0.01	0.24 ± 0.01	0.19 ± 0.01	0.12 ± 0.01

[a] Each group of five experimental groups, consisting of four immune syngeneic animals, was given i.v. 10^4, 10^5, 10^6, and 10^7, and 10^8 thymus cells/animal, respectively, at the time of tumor cell inoculum (1×10^6).

[b] Thymus cells were obtained from 20 syngeneic animals 7 days after tumor cell inoculation (1×10^6).

[c] NS, not statistically significant.

TABLE IV

Distribution of IS cells in lymphoid organs of TBA

Group No.[a]	Lymphocytes[b]	Tumor Size in cm²			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± S.E.			
I	Peripheral circulating lymphocytes	0.20 ± 0.01 (NS)[c]	0.26 ± 0.02 (NS)	0.20 ± 0.01 (NS)	0.18 ± 0.01 (NS)
II	Bone marrow cells	0.39 ± 0.01 ($p < 0.001$)	0.35 ± 0.01 ($p < 0.001$)	0.32 ± 0.01 ($p < 0.001$)	0.22 ± 0.01 ($p < 0.01$)
III	Draining lymph node cells[d]	0.37 ± 0.01 ($p < 0.001$)	0.32 ± 0.01 ($p < 0.001$)	0.25 ± 0.01 ($p < 0.01$)	0.22 ± 0.01 ($p < 0.02$)
IV	Spleen cells	0.38 ± 0.01 ($p < 0.001$)	0.46 ± 0.01 ($p < 0.001$)	0.51 ± 0.02 ($p < 0.001$)	0.34 ± 0.01 ($p < 0.001$)
V	Thymus cells	0.42 ± 0.02 ($p < 0.001$)	0.41 ± 0.01 ($p < 0.001$)	0.39 ± 0.01 ($p < 0.001$)	0.29 ± 0.01 ($p < 0.001$)
VI	Control Hanks' solution	0.20 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.13 ± 0.01

[a] Each group consisted of five immune syngeneic animals, each animal received s.c. 1×10^6 tumor cells.

[b] Lymphocytes (4×10^7) from various lymphoid organs were given intravenously into each group of immune syngeneic animals at the time of tumor cells inoculation.

[c] NS, not statistically significant.

[d] These cells consisted of a mixture of cells from cervical, axillary and inguinal lymph nodes.

TABLE V

IS cells in bone marrow

Group No.[a]	Treatment[b]	Tumor Size in cm²			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± S.E.			
I	BM cells of TBA non-treated	0.55 ± 0.002 ($p < 0.001$)	0.44 ± 0.01 ($p < 0.001$)	0.32 ± 0.004 ($p < 0.001$)	0.23 ± 0.003 ($p < 0.001$)
II	BM cells of TBA[c] treated with anti-θ and C	0.37 ± 0.01 (NS)[d]	0.20 ± 0.01 (NS)	0.15 ± 0.02 (NS)	0.11 ± 0.01 (NS)
III	Control Hanks' solution	0.31 ± 0.01	0.26 ± 0.01	0.18 ± 0.01	0.12 ± 0.002

[a] Each group consisted of five immune syngeneic mice; each animal received s.c. 1×10^6 tumor cells.

[b] Each animal of each of the experimental groups received i.v. 5×10^7 bone marrow (BM) cells of TBA (non-treated) or 5×10^7 eq. bone marrow cells of TBA treated with anti-θ serum and guinea pig complement (C) at the time of tumor cell inoculation. In control group, each animal received i.v. equal volume of Hanks' solution.

[c] Bone marrow cells (BM) (1.3×10^9) collected from 50 tumor-bearing animals were equally divided into two parts. One part remained as non-treated BM cells and the other part was treated with anti-θ serum and guinea pig complement by the procedure described in *Materials and Methods*. The treatment with anti-θ serum and complement resulted in the elimination of 6% of bone marrow cells.

[d] NS, not statistically significant.

TABLE VI

Cortisone resistance of IS cells

Group No.[a]	Treatment	Tumor Size in cm²			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± S.E.			
I	Thymus cells (non-treated)[b]	0.35 ± 0.01 ($p < 0.02$)	0.44 ± 0.02 ($p < 0.005$)	0.33 ± 0.02 ($p < 0.005$)	0.30 ± 0.02 ($p < 0.001$)
II	Thymus cells (cortisone-treated)[c]	0.39 ± 0.02 ($p < 0.02$)	0.43 ± 0.02 ($p < 0.005$)	0.29 ± 0.01 ($p < 0.005$)	0.27 ± 0.02 ($p < 0.001$)
III	Spleen cells (non-treated)[b]	0.34 ± 0.004 ($p < 0.005$)	0.33 ± 0.002 ($p < 0.001$)	0.27 ± 0.01 ($p < 0.005$)	0.28 ± 0.01 ($p < 0.02$)
IV	Spleen cells (cortisone-treated)[c]	0.41 ± 0.02 ($p < 0.005$)	0.46 ± 0.02 ($p < 0.005$)	0.34 ± 0.02 ($p < 0.005$)	0.34 ± 0.02 ($p < 0.01$)
V	Control Hanks' solution	0.23 ± 0.01	0.22 ± 0.004	0.16 ± 0.003	0.16 ± 0.003

[a] Each group consisted of five immune syngeneic animals; each animal received s.c. 1×10^6 tumor cells.

[b] Thymus or spleen cells were obtained from the control group of eight non-treated syngeneic animals 7 days after tumor cell (1×10^6) inoculation. Each animal of group I received i.v. 4×10^7 thymus cells and each animal of group III received i.v. 6×10^7 spleen cells at the time of tumor cell inoculation.

[c] Thymus or spleen cells were obtained from ten syngeneic animals which had been treated each with 2.5 mg of hydrocortisone i.p. 2 days before and had received tumor cells (1×10^6) 7 days before. Each animal of group II was given i.v. 4×10^6 thymus cells and each animal of group IV was given i.v. 4×10^7 spleen cells for hydrocortisone-treated mice at the time of tumor cell inoculation.

TABLE VII

Distribution of IS cells in spleen cell fractions of TBA, separated by Ficoll density gradient centrifugation

Group No.[a]	Fractionated Spleen Cells of TBA[b]	Tumor Size in cm^2			
		Day 3	Day 5	Day 7	Day 9
		mean tumor area ± S.E.			
I	Cells in first layer	0.35 ± 0.01 ($p < 0.05$)	0.39 ± 0.01 ($p < 0.001$)	0.25 ± 0.01 ($p < 0.001$)	0.18 ± 0.002 ($p < 0.001$)
II	Cells in second layer	0.25 ± 0.01 (NS[c])	0.16 ± 0.07 (NS)	0.07 ± 0.02 (NS)	0.03 ± 0.01 (NS)
III	Cells in third layer	0.27 ± 0.01 (NS)	0.12 ± 0.01 ($p < 0.01$)	0.04 ± 0.01 ($p < 0.05$)	0 ($p < 0.02$)
IV	Cells in the bottom layer	0.22 ± 0.02 (NS)	0.08 ± 0.01 ($p < 0.01$)	0.04 ± 0.01 ($p < 0.05$)	0.01 ± 0.004 ($p < 0.05$)
V	Control Hank's solution	0.27 ± 0.01	0.20 ± 0.01	0.10 ± 0.01	0.04 ± 0.01

[a] Each group consisted of five immune syngeneic animals and each animal was given s.c. 1×10^6 tumor cells.

[b] 1.5×10^9 spleen cells obtained from ten syngeneic animals 7 days after tumor cell inoculation were fractionated by centrifugation on a discontinuous density gradient of Ficoll. The cells of the four layers were washed with chilled Hanks' solution. Each mouse in each of the four groups (I ÷ IV) received i.v. 5×10^6 viable cells at the time of tumor cell inoculation.

[c] NS, not statistically significant.

indicating that IS cells are cortisone-resistant and appear to be a relatively mature T cell population in terms of the ontogeny of T cells (14).

Cell fractionation on centrifugation through a discontinuous Ficoll density gradient revealed that immunosuppressive activity seemed to be associated with a low density cell population. However, on the basis of the results obtained so far, it is not possible to postulate definite relationships between "immunosuppressor T cells" and cytotoxic T cells (effector T cells) on the one hand, and helper T cells (amplifying T cells) on the other. The IS cells in TBA may represent distinct subpopulations of T cells or T cells in a given state of differentiation. The answers to these problems will hopefully emerge from further systematic experiments.

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CHAPTER 3

REGULATION OF THE IMMUNE RESPONSE TO TUMOR ANTIGENS

III. CHARACTERIZATION OF THYMIC SUPPRESSOR FACTOR(S) PRODUCED BY TUMOR BEARING HOSTS

CHAPTER 1 and 2

The contributions of Dr. A. Sehon were directed at the guidance of the direction of certain experiments as well as editing the manuscripts. The individual contribution of Dr. S. Fujimoto was the kinetics of IST appearance and disappearance; and demonstration that IST were present in the bone marrow.

The discovery was previously reported (1,2,3,4) that immunosuppressor cells, which regulate the immune response to tumor antigens, were present in the thymus, spleen and other lymphoid organs of tumor-bearing mice. These cells were shown to be capable of specifically suppressing the rejection of a tumor in syngeneic mice immune to the corresponding tumor. Since the suppressive activity of the thymus cells of tumor-bearing hosts (TBH) was entirely eliminated by treatment with anti-θ serum and complement, it was concluded that these immunosuppressor cells belong to a subset of T cells (4).

In an attempt to establish if these immunosuppressor T (IST) cells produce a soluble factor(s) capable of regulating the host's immune response to tumor antigens, cell-free extracts were isolated from the thymocytes of tumor-bearing hosts. Indeed, these extracts were shown to exert an identical immunosuppressive effect, to that of IST, on the rejection of the tumor in immune syngeneic animals. In the present article are described some of the properties of the immunosuppressive factor(s) (ISF) extractable from the thymocytes of TBH, which has been shown to share antigenic characteristics common to the K-end of the major histocompatibility complex (MHC) of the mouse.

MATERIALS AND METHODS

Animals: A/Jax, BALB/cJ, C3H/HeJ, B10.A, B10.BR and B10.D2 strain mice were purchased from Jackson Laboratories, Bar Harbor, Maine. The animals used were 8 to 12 weeks old except for the hyperimmune mice which were somewhat older. Albino rabbits were obtained from North American Laboratory Supplies, Gunton, Manitoba.

Tumor Cells: The sarcoma induced by methylchloranthrene (S1509a) in A/J mice was kindly donated by Dr. P. Wright of the National Institutes of Health, Bethesda, Md. For tumor propagation 10^5 sarcoma cells, grown in the peritoneum of tumor-bearing donors and harvested in the ascitic fluid, were transferred intraperitoneally into 12 week old mice or were cultured in RPMI 1640 medium (Grand Island Biological Co., Long Island, N.Y.) which was supplemented with 5% fetal calf serum (FCS). Sarcoma I (SaI) which was a related tumor to S1509a, which had been also induced by methylchloranthrene in A/J mice, was purchased from Jackson Laboratories. The cells of either sarcoma used in these experiments represented the population obtained after three *in vitro* subcultures. The characteristics of the lymphoma L1117 which arose spontaneously in an A/J mouse in this laboratory had been reported earlier (5); this tumor is of viral origin and is composed of medium sized lymphoma cells and carries the θ antigen. The anaplastic carcinoma 15091A (AC-15), originally a spontaneous mammary carcinoma in an A/J mouse, was purchased from Jackson Laboratories. The tumor lines of these two unrelated but syngeneic tumors, referred to as L1117 and AC-15, respectively, were maintained by i.p. transfer into 3 to 5 month old A/J mice *in vivo* and by culture in RPMI 1640 medium supplemented with 5% FCS.

Preparation of TBH: For the purpose of producing TBH a volume of 0.1 ml containing 1×10^6 S1509a cells was injected s.c. into the centre of the shaved back of the A/J mice, using a 21 gauge needle (Becton Dickinson and Co., Canada Ltd.).

Measurement of Size of the Tumor: The tumors were measured, after the removal of the hair with a depilating agent (Neet) with Vernier

calipers, in two directions perpendicular to each other as described in the previous study (1,2). All experiments were done at least in duplicate.

Preparation of Immune Animals (IA): For this purpose 1 x 10^6 S1509a cells were injected s.c. into the shaved back of A/J mice. Seven days later the tumor surrounded by an area of normal skin was surgically excised and edges of the skin around the wound were glued together with a nonirritating chemical adhesive, *i.e.*, Aron Alpha manufactured by Toa Synthetic Chemical Co.,Ltd., Tokyo, Japan). After a period of 14 days the animals received a second dose of 10^6 tumor cells which was rejected. Hence, these animals were considered to have produced immunity to this tumor.

Preparation of Thymus Cell Suspensions: Thymuses were carefully removed from S1509a TBH without the parathymic lymph nodes. After removal, the thymuses were pressed and ground gently in chilled Hanks' solution with cold Hanks' solution. The cell number was determined microscopically using a hemocytometer and their viability determined by the trypan blue dye exclusion technique.

Preparation of Cell-free Soluble Extracts of Thymocytes or Spleen Cells of TBH: Cell-free soluble extracts of 2 x 10^9 washed thymocytes or spleen cells obtained from fifty TBH in 4 ml of Hanks' solution were prepared by alternate snap-freezing at -78°C and thawing at 37°C, repeated four times, followed by ultracentrifugation at 100,000 g for 1 hour. For the elimination of low molecular weight substances the soluble extracts were dialysed against 1 liter of borate-buffered saline (BBS), pH 8.0, for 16 hours.

Absorption of Soluble Extracts: The soluble extract obtained from the washed thymocytes of mice bearing the 1509a sarcoma was divided into equal volumes and each volume was incubated with 10^8 washed cells of a given tumor line at 4°C for 1 hour with constant, gentle rotation. The tubes were then centrifuged at 200 x g for 8 min. and the supernatants were further centrifuged at 15,000 rpm for 60 min. in the Sorvall RC2-B centrifuge (Newtown, Connecticut, U.S.A.).

Gel Filtration: The soluble extract obtained from the washed thymocytes of TBH was subjected to gel filtration through a column (2.5 x 100 cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) and fractionated by the upward flow of BBS at a flow rate of 18 ml/hr. Arbitrarily, the eluates were pooled into three fractions, corresponding to the fractions of normal mouse serum proteins with sedimentation coefficients of 19S, 7S and less than 4S which was used for calibration of the column, and the three fractions were then concentrated to the original volume by negative pressure dialysis.

Preparation of Rabbit Anti-mouse $F(ab')_2$ Serum: Antiserum to mouse $F(ab')_2$ was produced in rabbits by three intramuscular bi-weekly injections of 680 µg of $F(ab')_2$ fragments of mouse IgG_2 in Freund's complete adjuvant (FCA). For this purpose $F(ab')_2$ fragments were prepared in the following manner. Fifty ml of pooled whole mouse serum was treated with ammonium sulphate at 50% saturation and the precipitate was redissolved in BBS and this process was repeated three times. The globulins so precipitated were dissolved in 5 ml of saline and dialysed against BBS in the cold room and the IgG_2 fraction was isolated by preparative agar block electrophoresis in barbital buffer

(pH 8.6, μ = 0.1), as described in a previous study (5). The $F(ab')_2$ fragments were then prepared by the method of Nisonoff (6) with slight modifications. Briefly, 20 mg of IgG_2 was incubated with 0.6 mg of pepsin (Sigma Chemical Co. of St. Louis, Mo.) for 16 hours at 37°C in acetate-HCl buffer, pH 4.0, and the enzymatic digestion was stopped by adjusting the pH to 8.0 with 1 N NaOH; the reaction mixture was then dialysed against BBS. The $F(ab')_2$ fragments were then freed of any undigested globulins by gel filtration through a Sephadex G-200 column.

Preparation of Alloantisera and Congenic Antisera Directed to Antigens Coded by the H-$2K^k$ and H-$2D^d$ loci of MHC: For the preparation of alloantisera directed primarily to the antigens coded by the H-2 complex, BALB/cJ and C3H/HeJ mice were immunized with lymphoid cells of A/J mice to produce anti-H-$2K^k$ and I-A^k, or anti-H-$2D^d$ sera respectively, and for the preparation of congenic antisera to H-$2K^k$ and I^k or H-$2D^d$ loci of the MHC, B10.D2 and B10.BR mice were immunized with lymphoid cells of B10.A mice. For this purpose, spleens and lymph nodes of either seven A/J or seven B10.A mice were removed and teased to single suspensions. The first injection into the appropriate strain of mice consisted of an inoculum of 5×10^6 cells emulsified in FCA, into the footpads of 50 recipient mice. After two weeks, the recipients were given seven weekly injections of 5×10^6 of the antigenic cells intraperitioneally (i.p.). Thereafter, the immunized mice were partially bled from their tails prior to every subsequent injection. The sera of each group of recipient mice were pooled separately and heated at 56°C for 30 min. The cytotoxic antibodies

from these sera were determined by the ^{51}Cr release assay using as targets ^{51}Cr-labeled A/J or B10.A spleen cells, respectively (7). Fro 50% lysis of 10^5 of the appropriate target cells, the cytotoxic titers of the BALB/cJ and C3H/HeJ anti-A/J sera were 1:1280 and 1:320, respectively, and the titers of the B10.BR and B10.D2 anti-B10.A sera were 1:320 and 1:1280, respectively.

Preparation of Rabbit Antiserum to the Immunosuppressive T Cell Factor: Since the immunosuppressive factor(s) (ISF) from thymocytes of TBH was shown to bind specifically to the corresponding tumor cells, 2 ml of the soluble extract from 1×10^8 thymocytes of animals bearing the S1509a tumor was incubated with 5×10^6 S1509a cells at 4°C for 1 hour with constant, gentle stirring. The tumor cells were then washed with chilled Hanks' solution four times and resuspended in 2 ml of Hanks' solution and then emulsified with an equal volume of FCA . Two ml of the emulsified cell suspension were injected into the four footpads of each of two rabbits and the animals received thereafter two additional sets of injections, at two-week intervals, of the same volume of the emulsified cell suspension into their backs in multiple sites. Ten days after the third series of injections blood was collected by heart puncture and the sera were separated by centrifugation. The sera so collected were extensively absorbed with S1509a cells and tested by the cytotoxic assay after each absorption. Absorption was considered complete when these sera were devoid of cytotoxic activity to S1509a cells; these antisera will be referred to as anti-ISF sera.

Preparation of Reverse Immunosorbents: For insolubilization of antibodies to mouse $F(ab')_2$ fragments, a reverse immunosorbent was prepared by coupling the 7S globulins of the rabbit anti-mouse $F(ab')_2$ serum to CNBr activated Sepharose 4B by the method of Cuatrecasas (8) with some modifications. The 7S globulins were isolated from the whole serum by gel filtration on a Sephadex G-200 column. Sepharose 4B (40 ml) was washed several times with 0.1 M carbonate buffer, pH 9.0, and was resuspended in 40 ml of carbonate buffer. To this suspension was added 4 gm of cyanogen bromide (CNBr), dissolved in 40 ml of distilled water, and the pH was maintained at 11.0 for 8 minutes by dropwise addition of 4 N NaOH. The CNBr activated Sepharose was then washed within 5 minutes with 1500 ml of 0.1 M carbonate buffer using a Buchner funnel. To 20 ml of the activated Sepharose, resuspended in 0.1 M carbonate buffer, 20 mg of rabbit anti-mouse $F(ab')_2$ globulin was added and the mixture was stirred gently at 4°C for 24 hours. The resulting immunosorbent, denoted as Rb anti-m $F(ab')_2$ immunosorbent, was then washed with 0.01 M phosphate-buffered saline (PBS), pH 7.2, (until the optical density of the effluent at 280 nm was less than 0.01) and used for filling a small glass column.

To test the binding capacity of the Rb anti-m $F(ab')_2$ immunosorbent, 1 - 2 mg of purified mouse IgG_2 labeled with ^{125}I by the chloramine T method (13) was passed through the immunosorbent; any free radioactive iodine which may have been present in the sample of ^{125}I-IgG_2 was removed by prior filtration of the labeled globulin through a Sephadex G-25 column. The total amount of IgG_2 bound by the immunosorbent, calculated in terms of the radioactivity retained on the column, was

estimated to be close to 90%. The IgG_2 fraction was then eluted with a solution of glycine-HCl, pH 2.8, and the eluates were quickly neutralized with 0.1 N NaOH and concentrated to the original volume by negative pressure dialysis against BBS. The column was extensively washed thereafter with 0.01 M PBS for reuse in future experiments.

Other reverse immunosorbents were prepared in a similar manner, *i.e.*, by insolubilization of the globulin fractions of BALB/cJ anti-A/J, C3H/HeJ anti-A/J, B10.BR anti-B10.A, B10.D2 anti-B10.A antisera, and of normal serum; the last immunosorbent served for control experiments. Only crude globulin fractions precipitated with ammonium sulfate at 50% saturation were used for the preparation of these immunosorbents. As before, glycine-HCl buffer, pH 2.8, was used for elution.

Enzymatic type C from Streptococcus Griseus, 100 µg/ml) and bovine pancreatic ribonuclease (RNase A, 100 Kunitz units/mg) were purchased from the Sigma Chemical Co., St. Louis, Mo. To 1 ml volumes of the extract isolated from 2×10^9 thymocytes of TBH were added pronase (100 µg/ml) and RNase (50 µg/ml) and the mixtures were incubated for 1 hour at room temperature and pH 7.0. The digests were then centrifuged at 100,000 x g for 1 hour and the supernatants were diluted as required with cold BBS (9).

Statistical Analysis: To determine the level of significance, the experimental results were subjected to statistical analysis using the student's t-test. For each experiment, groups of four to five mice were used.

RESULTS

The Suppressive Effect of the Cell-free Soluble Extract of the Thymocytes of TBH on the Rejection of Tumor by Immune Mice: The possibility that the inhibition of tumor rejection in IA by thymocytes of TBH, which had been previously observed (1,2), was due to a subcellular thymic component(s) was examined. For this purpose, 2×10^9 thymocytes of mice bearing the S1509a tumor were used to prepare a cell-free extract. Five daily i.v. injections of the extract, corresponding to 1×10^7 thymocytes per injection, were administered to each mouse in the experimental group, beginning at the time of tumor inoculation. It is evident from the results plotted in Fig. 1 that the thymocyte extracts had the capacity to markedly suppress the tumor rejection. In fact the suppressive capacity of the extract was equivalent to that of the intact thymocytes from TBH (1,4) and was statistically significant on days 3, 7 and 13 after challenge of IA ($p < 0.025$). The extract of TBH spleen cells has also been shown to have similar suppressive activity (1,24).

The data in Fig. 1 also demonstrate the specificity of the thymic extract for a given tumor. Thus, whereas the suppressive capacity of the extract was depleted by treatment with 1×10^8 S1509a tumor cells harvested after three subcultures, the absorption of the extract with the unrelated syngeneic L1117 and AC-15 tumors did not decrease its suppressive activity. Furthermore, it was shown in earlier experiments (Mark Greene, unpublished observations) / that the thymus extracts of normal A/J mice or of A/J mice bearing the unrelated AC-15 tumor did not affect the growth of S1509a cells in A/J mice, which had been hyperimmunized to the S1509a tumor.

Hence, it may be concluded that the thymocytes of TBH possessed a factor capable of suppressing the immunological rejection of the tumor in preimmunized syngeneic animals and of binding specifically to the growing tumor.

Separation of the Soluble Immunosuppressive Factor(s) by Sephadex G-200 Gel Filtration: As is evident from Fig. 2, the fractionation of the extract of thymocytes of TBH by filtration through Sephadex G-200 gave rise to fractions (detected by their absorbancy at 280 nm) extending over a wide range of molecular sizes, corresponding to those of serum proteins with sedimentation coefficients between 19S and 4S. The individual eluates were, therefore, pooled arbitrarily into 3 fractions, as indicated in Fig. 2, and were concentrated to the original volume of the extract. Each of these 3 fractions at a dose of 45 µg per 0.1 ml was administered i.v. for 5 consecutive days into preimmunized mice beginning on the day of inoculation of tumor cells. From the results shown in Fig. 2, it is clear that Fraction III (*i.e.*, the fraction trailing behind the region corresponding to that of serum albumin and having an apparent molecular size smaller than that of albumin) was endowed with suppressive activity. The suppressive effect of Fraction III was statistically significant on days 4, 8 and 12, *i.e.*, $p < 0.025$. As shown below, this fraction had properties normally associated with proteins and, therefore, its concentration was estimated in terms of its optical density at 280 nm on the assumption that 1.5 O.D. unit corresponded to 1.0 mg protein per ml.

Specificity of Fraction III: As for the whole extract of thymocytes of TBH, the specificity of the reaction of Fraction III with S1509a

cells was assessed by treating equal volumes of it with 10^8 cells of different tumor lines, *i.e.*, AC-15, SaI and S1509a cells. It is evident from the data listed in Table I that absorption only with the homologous tumor cells depleted this fraction of its ISF, confirming thus again that the factor(s) responsible for the enhanced tumor growth in IA was indeed tumor specific.

Exposure of Fraction III to Enzymatic Degradation: In order to establish the chemical nature of the ISF, Fraction III was treated with pronase and RNase as described earlier and the supernatant, after exposure to these enzymes, was examined for suppressive activity. From the data listed in Table II, it is obvious that, whereas, RNase did not affect the suppressive activity of Fraction III, treatment with pronase resulted in a significant loss of activity. Hence, it may be considered that the factor responsible for the suppressive activity of Fraction III was, at least in part, a protein. It is also worth mentioning that the suppressive activity of Fraction III was lost on prolonged storage or on heating at 56°C for 1 hour.

Treatment of Fraction III with the Rb Anti-m $F(ab')_2$ Immunosorbent: In view of the specificity of the reaction of ISF with S1509a cells and in view of the relatively low molecular weight of the ISF (*i.e.*, lower than that of intact immunoglobulins), the possibility that this factor might represent an immunoglobulin fragment was examined. As stated under 'Materials and Methods', the efficiency of this reverse immunosorbent was of the order of 90%. Fraction III was applied onto the immunosorbent column and the effluent was concentrated to the original volume and tested for suppressive activity in the usual manner, *i.e.*, 45 µg of the effluent was administered daily for 5 days into hyperimmune

mice which received along with the first dose of the effluent 1×10^6 S1509a tumor cells. The results of this experiment demonstrated that the ISF present in Fraction III had not been retained on this immunosorbent and, therefore, one may conclude that this factor was not a conventional immunoglobulin. In this connection it is worth noting that the sera of TBH did not have any significant suppressive activity (4).

Test for Ability of C3H/HeJ anti-A/J, BALB/c anti-A/J, B10.D2 anti-B10.A and B10.BR anti-B10.A Sera to Neutralize the Effect of ISF: On the basis of the properties established so far for the ISF, the possibility that it was a product of the MHC (1) of the mouse was investigated, *i.e.*, it was suspected that the ISF may be coded or linked to the products of D or the K end of the H-2 locus of A/J mice. Therefore, it was expected that the C3H/HeJ anti-A/J serum or the BALB/c anti-A/J serum, which would contain antibodies to the product(s) of the D end and K end, respectively, may combine with the ISF and neutralize its effect. The description of the preparation of these antisera and of the corresponding reverse immunosorbents was given earlier. From the data listed in Table III, it is evident that whereas the C3H/HeJ anti-A/J reverse immunosorbent had no effect on the suppressive capacity of Fraction III, the BALB/c anti-A/J reverse immunosorbent depleted Fraction III of its suppressive activity. Moreover, the ISF could be recovered from the reverse immunosorbent by elution with 3 M NaSCN and was found to be effective even at a dose of 15 µg administered on 5 consecutive days. On the other hand, elution with glycine-HCl, pH 2.8, did not result in the recovery of the factor; this evidence suggests that the ISF is labile

at low pH.

By the use of reverse immunosorbents prepared with congenic antisera, it was possible to further substantiate the relationship of ISF to the MHC. Thus, as is evident from the data plotted in Fig. 3, whereas the interaction of Fr III with the B10.D2 anti-B10.A immunosorbent resulted in depletion of its immunosuppressive capacity, passage of Fr III through the B10.BR anti-B10.A antibody column did not affect the activity of ISF. Hence, one may conclude that the ISF was a product of or linked to a product of the K end of the MHC of A/J mice, that is related to the K^k, $I\text{-}A^k$, $I\text{-}B^k$ or $I\text{-}J^k$ subregions.

Biological activity of Anti-ISF Serum: The anti-ISF serum was elicited in rabbits, as described under Methods, using for immunization S1509a cells that had been employed for absorption of the ISF from the soluble extracts of thymocytes of A/J mice bearing the S1509a tumor. Extensive absorption of this serum with S1509a tumor cells resulted in removal of any detectable cytotoxic factors to S1509a cells or normal thymocytes. Treatment of thymocytes of TBH with this absorbed serum, in presence of guinea pig complement, did not lead to a significant loss in viability of these cells, *i.e.*, the cell viability was in the order of 90% as measured by the trypan blue dye exclusion technique. Similarly in control experiments normal rabbit serum had no detectable cytotoxic effect on thymocytes of TBH. However, as demonstrated by the results given in Table IV, treatment of thymocytes of TBH with the extensively absorbed anti-ISF serum, led to the apparent blocking of the capacity of these cells to suppress the rejection of S1509a cells in mice which had been hyperimmunized to the S1509a tumor. Hence, it may be concluded that the ISF was

represented by a distinct moiety on the thymocytes of TBH and that its effect could be neutralized by the anti-ISF serum which had been elicited by immunization with S1509a tumor cells coated with ISF.

DISCUSSION

The ability of the immune murine host to reject a methylchloranthrene-induced fibrosarcoma was previously shown to be amenable to suppression by the adoptive transfer of thymus-derived lymphocytes of mice bearing that same tumor (1-4,15,17). The suppressor cells in question were shown to be sensitive to treatment with anti-θ serum (2,24), to be steroid resistant, non-circulating and to be associated with low density thymocytes (2,3,24).

The results of the present study demonstrate that the factor(s) responsible for the biological activity of IST cells can be liberated by mechanical disruption of thymocytes of TBH and that the soluble extract of the IST cells is capable of counteracting *in vivo* the potential of immune animals to reject the same tumor. Furthermore, it was shown that this thymic ISF was endowed with immunological specificity inasmuch as it could be sequestered from solution by absorption with the corresponding tumor (*i.e.*, 1509a sarcoma cells), but not by other syngeneic tumors.

From the elution pattern of the soluble extract of IST cells on Sephadex G-200, it was obvious that the suppressive factor was associated with a moiety present in fraction III, which had the filtration properties corresponding to a molecular size smaller than that of mouse serum albumin (*i.e.*, less than 6.7×10^4 daltons).

Fraction III proved immunosuppressive in IA and was depleted of its suppressive activity on absorption with only the homologous S1509a tumor cells. The suppressive factor in Fraction III was inactivated at low pH, by heating at 56°C for 1 hour and by treatment with pronase; hence, it is inferred that the ISF is at least in part a protein.

In view of the specificity of the interaction of the ISF with the homologous tumor cells only, the possibility that it was an immunoglobulin or a Fab-like fragment was investigated with the aid of the Rb anti-m $F(ab')_2$ reverse immunosorbent. However, this experiment clearly demonstrated that this factor did not possess antigenic determinants common to mouse immunoglobulins. This result is in accord with the previous demonstration (1,4,15,24) that sera of TBH were devoid of suppressive activity and further strengthens the view that the ISF isolated in this study is not an ordinary immunoglobulins. Nevertheless, in view of the specific combination of ISF with the homologous tumor, it is conceivable that ISF is a product of V genes (26). The specific binding of the ISF to S1509a cells was exploited for the preparation of the anti-ISF serum, which in turn was capable of interacting with the ISF produced by IST cells and of, thus, neutralizing their immunosuppressive effect *in vivo*. Moreover, in view of the specificity of this binding, one may suggest that the observed immunosuppression of hyperimmunized mice by IST cells, or by the ISF produced by these cells, is due to the inactivation or elimination, or suppression of the generation of killer T cells either by complexes of tumor antigens and the homologous ISF or by the simultaneous interaction of killer T cells with suppressor cells,

bearing ISF on their surface, and tumor cells. However, since recent evidence (30, Mark Greene and Baruj Benacerraf, unpublished observations) have clearly shown that suppressor factors may themselves stimulate the production of suppressor cells, in certain situations, it is also conceivable that ISF in conjunction with tumor antigen is endowed with a similar capacity, and leads to the generation of other biologically active suppressor cells which limit the cytolytic potential. The finding that the reverse immunosorbent containing insolubilized allo-antibodies directed at products of the K-end of the H-2 locus of A/J mice depleted the thymus extract of TBH of its immunosuppressive activity and that the ISF could be eluted off this immunosorbent, suggested that the ISF was either a product of the MHC or a cell surface constituent linked to MHC. In fact, the experiments with the antisera produced in congenic strains of mice clearly demonstrate that the ISF shared the antigenic determinants of the K-end of the MHC of the mouse. Since the ISF was removed by this sera, which has been shown to manifest anti-I-J activity (Mark Greene, unpublished observations) it is intriguing to speculate that the suppressor factor may be coded by this subregion, as has been the case of other supressor factors defined recently by Tada et al. (18). In addition to the immunochemical relationship between ISF and other murine factors, one may also examine the functional relationship(s) between these factors, which play a crucial role in the regulation of the immune response. Functional description of thymic factors has been in terms of augmentation or suppression of immune responses. The present results are reminiscent of the thymic factor

described by Takemori, *et al.* (21) which demonstrated carrier-specific suppressor activity in relation to the hapten-specific antibody response. Also Taniguchi, *et al.* (27) and Tada and Taniguchi (28) as previously stated, have demonstrated that the antigen-specific suppressor factors are probably products of the I-region of the H-2. The similarities of ISF with the antigen-specific suppressor factor operative in the suppression of transfer of contact sensitivity are also quite remarkable (29 ISF also shares many of the properties of the suppressor factor elegantly defined by Theze, *et al.*(31). In contrast to these findings, Armerding *et al.* (23) as well as Munro, *et al.*(22) have isolated thymic factors which can augment the immune response.

It is also to be noted that there appears to exist a relationship between MHC of a host and its resistance or susceptibility to certain tumors (9-12, 25). The products of the MHC have also been shown to be linked to tumor antigens (5,14), to restrict cell elimination (16) to limit T cell proliferation (19) and to suppress cell interaction in the mixed lymphocyte reaction (20). To this list of MHC functions, one may also add its role in the suppression of cytolytic tumor elimination in animals immune to the tumor. However, the precise mechanism by which MHC related products determine the outcome of these various physiological manifestations remains to be elucidated.

TABLE I. SPECIFICITY OF IMMUNOSUPPRESSIVE ACTIVITY OF THE SOLUBLE FACTOR(S) OF FRACTION III

Group No.*	Treatment**	Tumor Size (mean tumor area cm^2 ± SEM)			
		Day 3	Day 5	Day 7	Day 9
Control	Hanks' solution	.28 ± .01	.26 ± .02	.22 ± .01	.16 ± .02
I	Fr. III, absorbed with S1509a cells	.26 ± .01 (n.s.)***	.25 ± .01 (n.s.)	.21 ± .01 (n.s.)	.16 ± .02 (n.s.)
II	Fr. III, nonabsorbed	.49 ± .03 ($p < .01$)	.51 ± .02 ($p < .001$)	.46 ± .02 ($p < .005$)	.38 ± .01 ($p < .005$)
III	Fr. III, absorbed with SaI cells	.54 ± .01 ($p < .001$)	.48 ± .02 ($p < .01$)	.43 ± .01 ($p < .005$	.37 ± .01 ($p < .005$)
IV	Fr. III, absorbed with AC-15	.54 ± .01 ($p < .001$)	.51 ± .02 ($p < .005$)	.46 ± .01 ($p < .001$)	.41 ± .02 ($p < .01$)

* All groups consisted of five syngeneic mice immune to 1509a sarcoma.

** Each mouse received i.v. 45 µg/day of the whole or absorbed Fr. III for 5 consecutive days, beginning on the day of challenge with 1×10^6 S1509a cells.

*** n.s. - not significant.

TABLE II. THE EFFECT OF TREATMENT OF FRACTION III WITH DIFFERENT ENZYMES

Group No. *	Treatment	Tumor Size (Mean Tumor area cm^2 ± SE)				
		Day 3	Day 5	Day 7	Day 9	Day 11
I	Fraction III alone	.42 ± .04 ($p < .005$)	.32 ± .04 ($p < .025$)	.32 ± .04 ($p < .05$)	.18 ± .02 ($p < .05$)	.16 ± .03 ($p < .025$)
II	Fraction III after treatment with RNase	.32 ± .04 ($p < .025$)	.29 ± .04 ($p < .05$)	.29 ± .03 ($p < .05$)	.16 ± .02 ($p < .1$)	.09 ± .01 (p.n.s.)
III	Fraction III after digestion with pronase	.17 ± .02 (n.s.)**	.16 ± .01 (n.s.)	.16 ± .02 (n.s.)	.12 ± .03 (n.s.)	.06 ± .03 (n.s.)
Control	Hanks' solution	.20 ± .03	.17 ± .03	.16 ± .03	.09 ± .04	.05 ± .03

* All groups consisted of 4 hyperimmune syngeneic animals.

II p.n.s. - probability not significant.

TABLE III. THE RELATIONSHIP OF FRACTION III TO THE MAJOR HISTOCOMPATIBILITY COMPLEX

Group No. *	Test Material	Tumor Size (Mean Tumor Area cm^2 ± SE) Day 3	Day 5	Day 7	Day 9
I	Fraction III untreated	.35 ± .02 ($p < .005$)	.49 ± .03 ($p < .005$)	.44 ± .03 ($p < .005$)	.37 ± .04 ($p < .005$)
II	Effluent after passage of Fraction III through mouse globulin immunosorbent **	.35 ± .02 ($p < .005$)	.45 ± .03 ($p < .005$)	.42 ± .02 ($p < .005$)	.34 ± .05 ($p < .05$)
III	Effluent after passage of Fraction III through C3H/HeJ anti-A/J reverse immunosorbent	.31 ± .02 ($p < .005$)	.49 ± .04 ($p < .005$)	.44 ± .03 ($p < .005$)	.27 ± .03 ($p < .05$)
IV	Effluent after passage of Fraction III through BALB/c anti-A/J reverse immunosorbent	.24 ± .03 (n.s.)**	.30 ± .04 (n.s.)	.27 ± .05 (n.s.)	.24 ± .03 (n.s.)
V	Eluate obtained with 3 M NaSCN from BALB/c anti-A/J reverse immunosorbent after interaction of Fraction III with this immunosorbent	.40 ± .02 ($p < .005$)	.47 ± .04 ($p < .005$)	.58 ± .05 ($p < .005$)	.55 ± .06 ($p < .005$)
VI	Eluted at pH 2.8 from BALB/c anti-A/J reverse immunosorbent after interaction of Fraction III with this immunosorbent	.24 ± .05 (n.s.)	.31 ± .06 (n.s.)	.23 ± .06 (n.s.)	.19 ± .04 (n.s.)
Control	Hanks' solution injected in lieu of Fraction III	.19 ± .02	.25 ± .02	.23 ± .03	.17 ± .03

* Each group consisted of 5 hyperimmune A/J mice.

** This immunosorbent was prepared by coupling mouse globulins to Sepharose 4B; it also served as an additional control to eliminate the possibility of the nonspecific uptake of the suppressive factor by proteins attached to a Sepharose matrix.

*** n.s. - not significant.

TABLE IV. ABILITY OF ANTI-ISF SERUM TO INHIBIT THE SUPPRESSIVE ACTIVITY OF THYMOCYTES OF TBH

Group No. *	Treatment of IA with**	Tumor Size (mean tumor cm^2 ± SE)				
		Day 3	Day 5	Day 7	Day 9	Day 11
I	1×10^8 thymocytes	.22 ± .03 ($p < .05$)	.23 ± .03 ($p < .05$)	.35 ± .05 ($p < .05$)	.47 ± .08 ($p < .05$)	.37 ± .07 ($p < .05$)
II	1×10^8 thymocytes of TBH pretreated with anti-ISF + complement	.17 ± .02 (n.s.)***	.13 ± .01 (n.s.)	.16 ± .04 (n.s.)	.24 ± .05 (n.s.)	.13 ± .02 (n.s.)
Control Δ	Hanks' solution	.12 ± .02	.14 ± .01	.20 ± .03	.24 ± .03	.17 ± .01

* Each group consisted of 5 hyperimmune syngeneic animals.

** In addition to the materials indicated in this column, all hyperimmune animals received s.c. the standard dose of 10^6 S1509a tumor cells.

*** n.s. - not significant.

Δ Normal rabbit serum and C' had no effect.

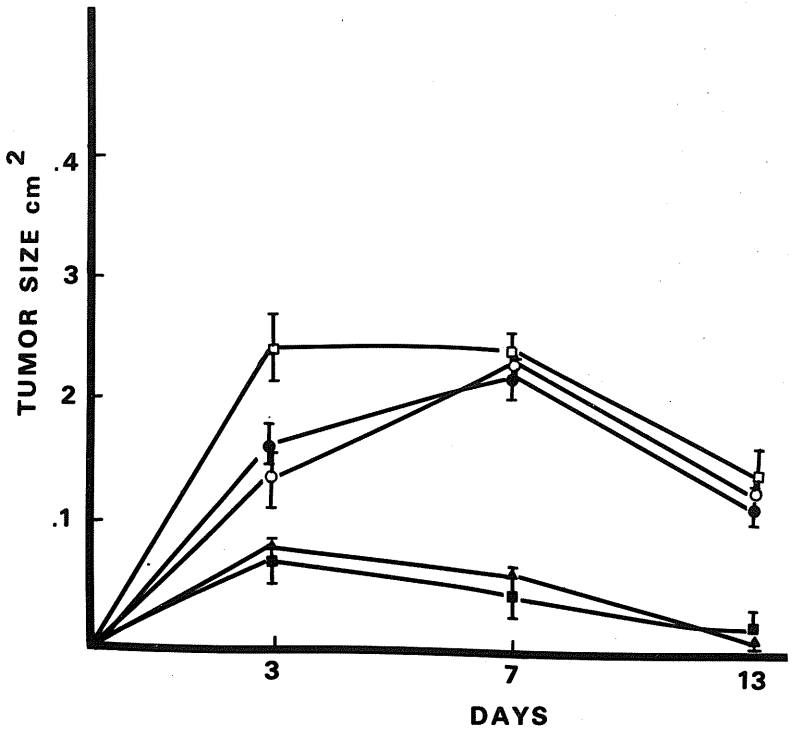

Figure 1

A/J animals hyperimmune to the S1509a tumor were challenged s.c. with a dose of 10^6 S1509a cells. The i.v. administration of the intact or absorbed thymic extract of the thymuses of mice bearing the S1509a tumor was begun at the time of challenge. The extract, equivalent to 10^7 thymus cells, was given each day for 5 days. Represented in this figure are resulted obtained with the whole thymus extract (□----□); extract absorbed with 10^8 L1117 cells (●----●), or with 10^8 AC-15 cells (o----o), or with 10^8 S1509a cells (▲----▲). The control group (■----■) received BBS.

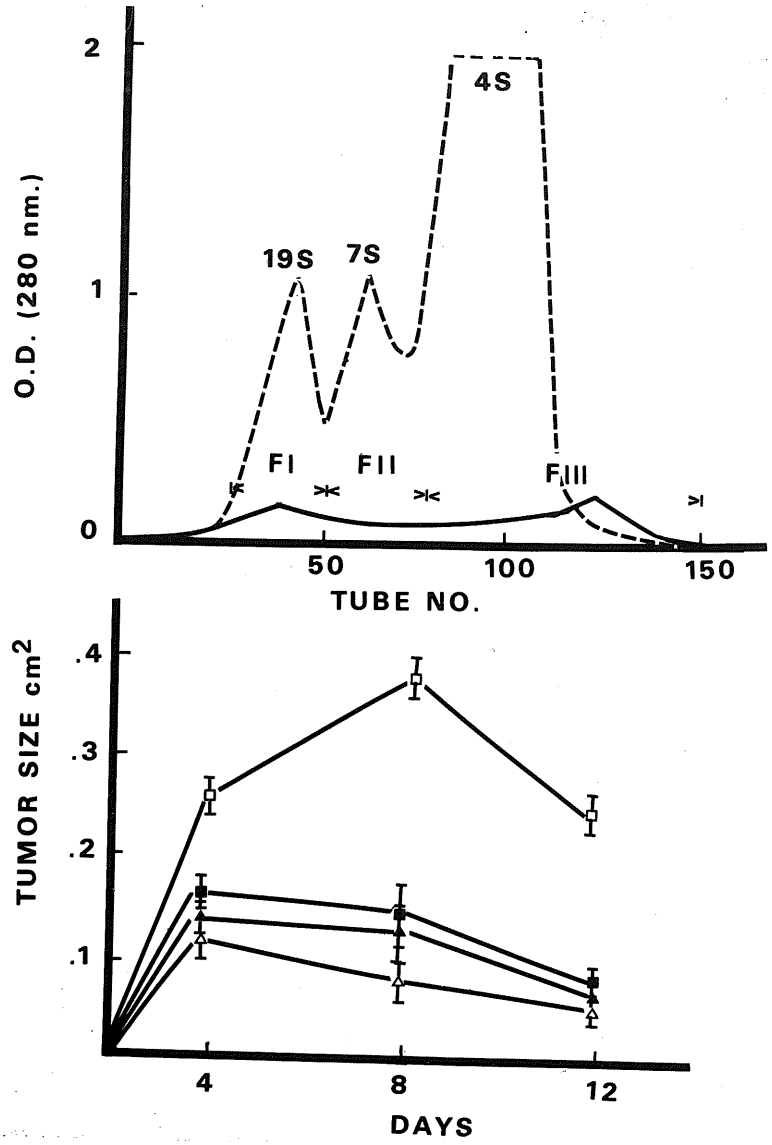

Figure 2

Upper diagram: The elution pattern at 280 nm of the thymus extract of TBH after filtration through a Sephadex G-200 column is indicated by the solid line; the elution pattern of normal serum of A/J mice on the same column is shown by the broken line. The eluates corresponding to the three fractions I, II and III were pooled and concentrated to the original volume of the extract and tested.

Lower diagram: The three fractions I, II and III were administered at a dose of 45 µg/day/mouse for 5 consecutive days into hyperimmune A/J mice beginning at the time of challenge with 10^6 S1509a tumor cells. Mice which received fractions I, II and III are indicated, respectively, by symbols (Δ----Δ), (▲----▲) and (□----□); control mice received BBS (■----■).

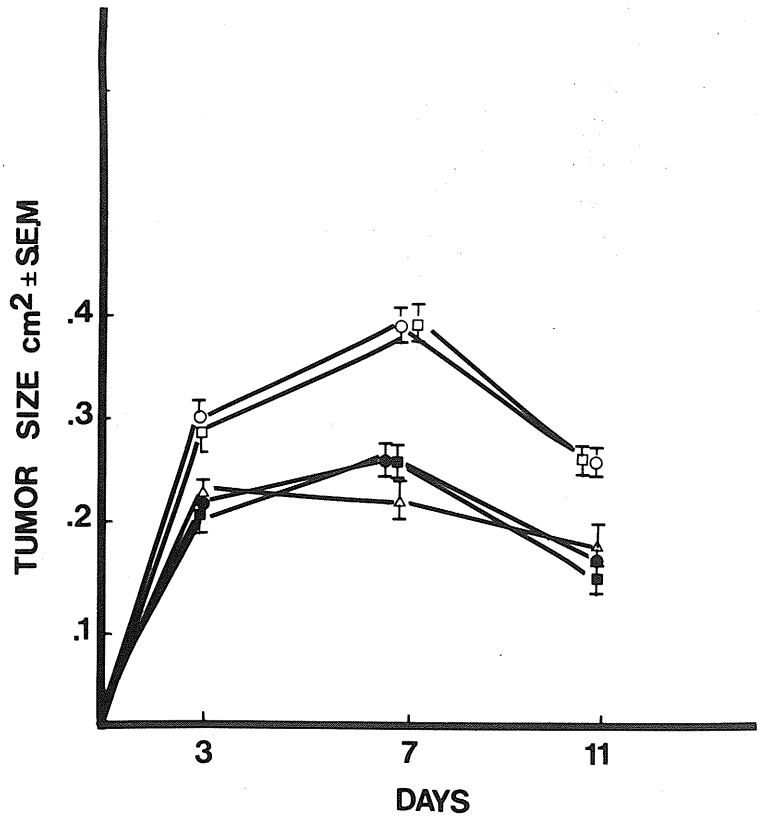

Figure 3

TBH thymocyte extract was divided into equal aliquots and passed through reverse immunosorbents or was absorbed with S1509a cells. The extracts after such treatment were administered at a dose of 45 µg/day/mouse for 5 consecutive days into hyperimmune A/J mice beginning at the time of challenge with 10^6 S1509a cells. Represented in this figure are the results obtained with untreated thymus extract (□----□), with thymus extract absorbed with S1509a cells (●----●), with thymus extract passed through a B10.BR anti-B10.A reverse immunosorbent (o----o), or with thymus extract passed through a B10.D2 anti-B10.A immunosorbent (Δ----Δ). The control mice received BBS (■----■).

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CHAPTER 3

The contributions of Dr. A. Sehon were directed at the guidance of the direction of certain experiments as well as editing the manuscripts.

CHAPTER 4

THE NON-ADAPTIVE REJECTION OF SMALL TUMOR INOCULA AS A MODEL OF IMMUNE SURVEILLANCE

Thomas's thesis (1) that it is a "universal requirement of multicellular organisms to preserve uniformity ot cell type", led him to suggest that the phenomenon of homograft rejection represented the primary mechanism for natural defense against neoplasia. Burnet (2) viewed this surveillance of lethal oncogenic mutations as a function of the thymus-dependent adaptive immune system. Prehn (3,4) and others (5,6,7) have criticized the Burnet model of immune surveillance because it is unable to account for several important observations: 1) the adaptive immune system, with only rare exception, is unable to eliminate incipient or induced tumor rather than functioning as a surveillance mechanism (4,6,7,8); 2) the immune response to tumors is as likely to result in immunosuppression and enhancement of tumor growth as the destruction of those tumors (6,8,9); and 3) the incidence of spontaneous tumors in athymic mice is insignificant (5). To explain these data one must either assume that surveillance does not exist, or one must postulate that surveillance exists in a form other than the thymus-dependent adaptive immune response. We have attempted to examine the hypothesis that surveillance exists and is a non-thymus dependent phenomenon by an experimental model in which the fate of a small tumor inoculum, simulating an oncogenic mutation, is examined after manipulation of the hosts immune response.

Several carcinogen-induced tumor lines, the 1509a fibrosarcoma, P-815-X2 mastocytoma and the SL2 lymphoma, as well as the spontaneous lymphoma L5178Y, when implanted subcutaneously in small numbers fail to produce death or a palpable tumor mass in a large proportion of syngeneic recipients (Table 1). To identify the number of injected

cells capable of forming a tumor mass, medium containing 50 cells of L5178Y or P-815-X2 was placed in a soft agar gel which allows the formation of single cell clones (10). Cloning efficiencies of up to 46% per inoculum were demonstrated after three weeks of culture (Table 1). The 1509a tumor cells were also shown, in repeated experiments, to grow well *in vitro*. Inocula of 10^2, 10^3 and 10^4 cells produced 5 x 10^4, 10^6 and 5 x 10^6 tumor cells respectively, in 14 days. The capacity of the small tumor inocula to grow *in vivo* was demonstrated after intraperitoneal implantation of the 1509a, L5178Y and P-815-X2 (Table 1). Despite the ability of 10^2 1509a to grow intraperitoneally, intranveous as well as subcutaneous challenge with that number of cells produced very few tumors (Table 1). This may reflect a distinct distribution of effector cells within the host.

The rejection of the small subcutaneous inocula, however, could be prevented by the simultaneous intravenous injection of papain digest or 3M KCl extracts of syngeneic tumor membrane (Table 2). Tumor frequency was 2.5 to 3.5 times higher than untreated controls or controls receiving allogeneic membrane at the optimal dose of membrane extract.

The existence of memory was assessed in mice surviving the initial challenge of a small tumor inoculum by rechallenging with a larger tumor dose which was observed in preliminary experiments to result in 100% mortality. The latency and survival rate of mice which had previously rejected the L5178Y and P-815-X2 inocula was no different from that of normal control mice (Table 1). No difference was observed in the survival rates when mice were inoculated with

tumor obtained from either *in vivo* peritoneal passage or *in vitro* tissue culture (Table 1). Similarly, mice which had rejected the 1509a in the primary challenge with the small inocula did not reduce either the growth rate or the mortality rate of the secondary challenge. It was also found that mice which had rejected 10^2 1509a cells had the same tumor incidence as untreated controls when rechallenged with the same small inoculum (Table 1).

We examined the effects of suppression of the immune response on the incidence of tumors. A/J mice can be made resistant to 1509a by surgical removal of the primary tumor, and the subsequent simultaneous administration of suppressor T cells and 1509a to these immune mice results in enhanced tumor growth (9). However, suppressor T cells given with a small inoculum of 1509a in a primary challenge did not result in any enhancement in the frequency of tumors or their growth rate. In addition, the simultaneous administration of suppressor T cells and the small primary tumor inoculum did not enhance the growth rate of the secondary challenge of 1509a (Figure 1).

We further examined the question of whether the rejection of small tumor inocula was a thymus-dependent adaptive immune phenomenon by subcutaneously implanting the 1509a and the L5178Y in their respective syngeneic hosts after adult thymectomy, lethal irradiation and bone marrow reconstitution. The frequency of tumors was slightly less in the immunodeficient mice than in the intact control mice, despite a much reduced survival rate when challenged with a large tumor dose (Table 3).

If the inoculation of a small number of tumor cells is an adequate model for an oncogenic mutation, the ability of immunodeficient

or immunosuppressed mice to reject them suggests that surveillance is not a thymus-dependent adaptive immune phenomenon. This thesis is also supported by the additional evidence that the rejection is not associated with memory.

The ability of syngeneic tumor membrane extracts to enhance tumor frequency suggests that the failure of tumor growth is due to a rejection mechanism with at least some specificity. It is also clear from our observations that one of the feature characteristics of this mechanism is the rapidity of its response, acting well before the tumor has reached a critical size. Since tumor growth is very rapid and the time required to reach this critical size is theoretically very small, it suggests that the recognition mechanism is functionally optimal in the normal animal and capable of acting before an adaptive immune response can be generated. Such pre-existing specific immune mechanisms have been identified in two forms in mammals; naturally occurring antibodies (11,12) and naturally occurring cytotoxic cells (13,14,15).

Natural antibodies with specificity for spontaneous or carcinogen-induced tumor, such as were used in the present study, have been found in normal mouse sera as well as sera from congenitally athymic (nu-nu) mice and are predominantly IgM (12,16). The role of IgM in anti-tumor immunity is not entirely clear, but it has been implicated in cell-mediated immunity to murine sarcoma virus (17) and mammary tumor virus induced tumors (18).

Naturally occurring cytotoxic cells have been demonstrated with well-defined specificities in *in vitro* assays of tumor immunity (13,14,15)

and are under genetic control of a strong H-2 linked factor in mice (19). These cells have been partially characterized and bear neither B nor T cell membrane antigens (13,14,20). Keissling *et al.* (20) have also recently demonstrated a correlation between *in vitro* cytolysis by naturally occurring cytotoxic cells and their ability to produce *in vivo* neutralization of an MSV lymphoma in a Winn assay. It is not clear at this time whether mice possess these effector cells in sufficient heterogeneity to account for the recognition of the diverse populations of tumors expected as a consequence of carcinogen-induced mutations, viral transformation, or tumor induction by other oncogens. However, the possibility that these effector cells are responsible for the rejection phenomenon we have observed must certainly be entertained.

We believe, therefore, that if immune surveillance of tumors exists, the possibility that it functions as a thymus independent, non-adaptive phenomenon must be considered. Although the nature of this type of a surveillance mechanism is not immediately apparent, its identification may be of considerable importance to tumor biology and immunotherapy.

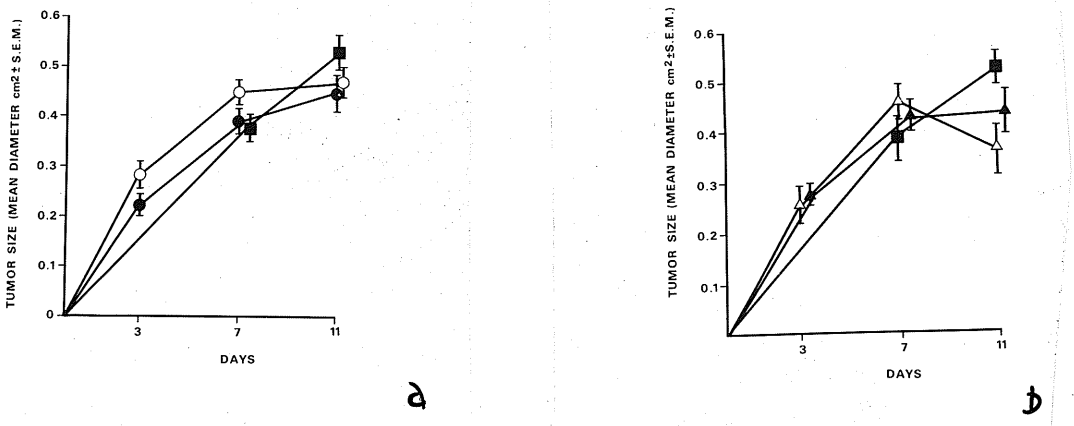

Fig. 1 Absence of memory after the rejection of a small 1509a tumour inoculum. *a*, Primary inoculation with 10^2 1509a subcutaneously was followed by a secondary challenge with 10^6 tumour cells 30 d later in the tumour-free mice. Suppressor T cells obtained from A/J thymus[9] were given intravenously, simultaneously with the 10^2 inoculum in a second group of mice. Control mice did not receive a primary inoculation before 10^6 tumour challenge. The tumour size was measured with a Vernier caliper in two diameters at right angles. Observations were made on five mice in each experimental group. (●) 10^6 1509a secondary challenge after rejection in 10^2 primary challenge; (○) 10^6 secondary challenge after injection of a primary challenge with 10^2 1509a and 4×10^7 suppressor T cells; (■) 10^6 1509a primary challenge. *b*, Experimental protocol is identical to *a* except the primary inoculation is 10^3 1509a.

Table 1 Mice rejecting small syngeneic inocula in a primary challenge fail to exhibit memory on secondary challenge

Experiment	Tumour	Route of adminstration§	Inoculum size	%Cloning efficiency¶ ±s.e.m.	Tumour incidence on primary challenge Number	%	Large inoculum second challenge** Latency‡ (d ±s.e.m.)	Survival (d ±s.e.m.)	Small inoculum second challenge†† Frequency Number	%
1.	L5178Y*	Subcutaneous	5×10^1	47 ± 5	4/15	26	—	—	—	—
2.	L5178Y*	Subcutaneous	5×10^1	25 ± 7	8/40	20	12.5 ± 2.0	35.4 ± 1.0	—	—
		Intraperitoneal	5×10^1	25 ± 7	15/15	100	—	—	—	—
		Control	0	—	0/15	0	13.5 ± 2.5	36.4 ± 2.2	—	—
3.	P-815-X2*	Subcutaneous	5×10^1	44 ± 2	11/40	27.5	8.0 ± 1.0	33.8 ± 1.3	—	—
		Intraperitoneal	5×10^1	44 ± 2	15/15	100	—	—	—	—
		Control	0	—	0/15	0	7.9 ± 1.0	32.1 ± 2.3	—	—
4.	L5178Y†	Subcutaneous	10^1	—	1/8	12.5	—	33 ± 2	—	—
		Subcutaneous	5×10^1	—	2/8	25	—	40 ± 3	—	—
		Subcutaneous	10^2	—	3/8	37.5	—	33 ± 5	—	—
		Control	0	—	0/5	0	—	45 ± 2	—	—
		Intraperitoneal	10^1	—	13/15	87	—	—	—	—
5.	P-815-X2†	Subcutaneous	10^1	—	4/8	50	—	38 ± 6	—	—
		Subcutaneous	5×10^1	—	2/8	25	—	39 ± 3	—	—
		Subcutaneous	10^2	—	8/8	100	—	—	—	—
		Control	0	—	0/5	0	—	41 ± 5	—	—
		Intraperitoneal	10^1	—	8/8	100	—	—	—	—
6.	SL2†	Subcutaneous	10^1	—	3/8	37.5	—	37 ± 6	—	—
		Subcutaneous	5×10^1	—	6/8	75	—	32 ± 5	—	—
		Subcutaneous	10^2	—	4/8	50	—	30 ± 7	—	—
		Control	0	—	0/5	0	—	35 ± 3	—	—
7.	1509a*	Subcutaneous	10^2	—	3/20	15	—	—	3/14	21
		Subcutaneous	10^3	—	5/20	25	—	—	2/10	20
		Subcutaneous	10^4	—	13/20	65	—	—	3/6	50
		Subcutaneous	10^5	—	20/20	100	—	—	—	—
		Intraperitoneal	10^2	—	5/5	100	—	—	—	—
		Intraperitoneal	10^3	—	5/5	100	—	—	—	—
		Intravenous	10^2	—	0/10	0	—	—	—	—

*Tumours obtained from *in vitro* cultures for both primary and secondary challenge.
†Tumours obtained from ascitic passage for both primary and secondary challenge.
‡Appearance of palpable tumour after subcutaneous inoculation.
§All inoculations were 0.1 ml of washed cells. Subcutaneous inoculations were made in mid-low back after removing the fur.
¶After thorough washing, the tumour was resuspended in Fischer's medium containing 15% foetal calf serum. Aliquots of 2.0 ml containing 5×10^1 cells were added to 3 ml of 2% Noble special agar at 44° C. After thorough mixing, the suspension was placed in a 37° C incubator at 5% CO_2 in a humid atmosphere. Visible clones were counted after 2 weeks in culture. Cloning efficiency was calculated from a minimum of 10 replicates.
**An aliquot of 10^5 tumour cells in 0.1 ml Fischer's medium was inoculated subcutaneously in the same site as the primary challenge.
††Tumour frequency was assessed 25 d after subcutaneous challenge with the same number of tumour cells as the primary inoculum.

Table 2 Enhancement of tumour frequency after subcutaneous implantation of small tumour inocula by the intravenous injection of syngeneic tumour membrane preparations

Membrane preparation Tumour*	Amount† (μg)	Tumour inoculum		Tumour frequency‡ Number	%
L5178Y	0.1	5×10^1	L5178Y	7/16	44
L5178Y	1.0	5×10^1	L5178Y	12/23	52
L5178Y	10.0	5×10^1	L5178Y	6/16	37
—	0	5×10^1	L5178Y	7/35	20
1509a	1.0	5×10^1	L5178Y	2/12	17
1509a	0.1	10^2	1509a	4/10	40
1509a	1.0	10^2	1509a	7/10	70
1509a	10.0	10^2	1509a	5/10	50
—	0	10^2	1509a	2/10	20
L5178Y	1.0	10^2	1509a	0/5	0

*Papain digest of L5178Y membrane was prepared by the method of Maramatsu *et al*[21]. The membrane digest was centrifuged at 25,000 r.p.m. for 10 min and placed on a Sephadex G-200 column. A single protein peak of low molecular weight was observed. The 3 M KCl membrane extract of 1509a was prepared by the method of Forbes *et al.*[22].

†$1A = 0.7142$ mg ml^{-1} at E_{280}

‡Tumour frequency was assessed 25 d after the implantation of the subcutaneous tumour inoculum. The data represent a total of three experiments for the L5178Y and two experiments for 1509a.

Table 3 Frequency of tumours and mortality of adult thymectomised, lethally irradiated and bone marrow reconstituted mice (AT × BM) challenged with small and large tumour inocula

Experiment	Tumour	Inoculum size	Recipient†	Tumour‡ frequency No.	%	Survival (d ± s.e.m.)
1.	L5178Y*	5×10^1	Normal DBA/2	3/15	20	> 40
		5×10^1	AT × BM DBA/2	0/15	0	—
2.	L5178Y	10^5	Normal DBA/2	19/19	100	35 ± 2§
		10^5	AT × BM DBA/2	14/14	100	18 ± 1
3.	1509a	10^2	Normal A/J	0/5	0	—
		10^2	AT × BM A/J	0/5	0	—
		10^3	Normal A/J	2/5	40	—
		10^3	AT × BM A/J	1/5	20	—
		10^6	Normal A/J	5/5	100	—

*Cloning efficiency of the L5178Y was 47 ± 5% per inoculum.

†AT × BM mice received 800 rad irradiation 4 weeks after adult thymectomy followed by 2.5×10^7 washed bone marrow cells intravenously. Mice were challenged with tumour 3–4 weeks later.

‡Tumour frequency was assessed at 30 d after inoculation of tumour.

§$P < 0.001$

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CHAPTER 5

CHARACTERISTICS OF A NON-ADAPTIVE THYMUS INDEPENDENT SURVEILLANCE MECHANISM

The thymus-dependent adaptive immune system has generally been assumed to be the mechanism which is responsible for the elimination of lethal oncogenic mutations (1). Recently we have provided evidence for a non-adaptive surveillance (NAS) mechanism (2). The characteristic features of this mechanism included the ability to eliminate small tumor inocula presented to the host by a variety of routes, the absence of memory after successful rejection of these inocula and no demonstrable deleterious effect of T cell ablation or immunosuppression on the response of the host. With the additional observation that the simultaneous subcutaneous (s.c.) inoculation of small tumor inocula and intravenous administration of solubilized syngeneic tumor membrane led to a consistently increased number of successful tumor takes, we concluded that the effector mechanism can be blocked by syngeneic membrane antigens and that its activation occurs in the absence of an adaptive immune response.

It is of obvious importance to document the evolution of responses that are initiated from an oncogenic mutation. Secondary responses to tumors have been shown to be T cell dependent (3) and, for some tumors, can be modulated by suppressor T cells (4,5). Analysis of the particular manner by which a primary tumor bearing host responds to a tumor may reveal that a growing tumor evokes different responses at different times in its history. In the following communication we have analyzed some of the characteristics of the primary response to small and large tumor inocula and the factors which influence it.

Mice: The 6 to 8 week old A/J and DBA/2 mice which were used in most experiments were purchased from Jackson Laboratories, Bar Harbor, Maine. Age dependent experiments used 44 - 52 week old mice, while hyperimmune mice were generally 12 - 16 weeks old.

Tumors: The Sal509a (2,4,5) and the L5178Y (2) have been described previously.

Preparation of Suppressor T cells: A/J mice were inoculated with 1 x 10^6 Sal509a cells in 0.1 ml s.c. into the center of the shaved back and 7 days later suppressor cells were obtained from the thymuses of these tumor bearing hosts (TBH). The thymuses were pressed and ground in chilled Hank's using glass grinders to release single cells from fibrous tissues. The cells were passed through a 150 mesh platinum screen and washed three times with cold Hank's solution. The cell number was determined microscopically using a hemocytometer and their viability determined by the Trypan Blue dye exclusion technique.

Simultaneous Inoculation of Large and Small Tumor Inocula The entire back of a mouse was shaved and on one half a large inoculum of cells was injected subcutaneously in a volume of 0.1 ml and the other half, a small tumor inocula was administered in the same volume.

Assessment of Suppressor Cell Activity It has been shown that thymocytes from hosts bearing actively growing tumors can suppress the rejection of a challenge of 10^6 Sal509a cells in immune animals (6,7). After a period of 7 - 10 days following the administration of

large and small tumor inocula, the thymuses were removed and cell suspensions were made. Thymocytes (3.5×10^7) were transferred I.V. to immune animals which were then rechallenged with 10^6 Sa1509a cells s.c. Tumor growth was measured with vernier calipers and the statistical analysis of growth rates was performed using the student's t-test.

Preparation of Soluble Tumor Membranes Soluble tumor membranes were prepared by the use of 3M KCl as previously described (2) or by the use of limited papain digest (2).

RESULTS

THE INCIDENCE OF TUMOR AFTER LOW DOSE INOCULA:

In order to establish that small tumor inocula are actively eliminated, small amounts of solubilized tumor membranes were intravenously administered at the same time as the subcutaneous inoculation of tumor cells. This procedure produced a significant increase in the incidence of tumor take at 21 days (Table 1). It is also evident that T cell deprivation by adult thymectomy, lethal irradiation followed by bone marrow reconstitution had no discernible effect on the tumor frequency. The results lead to the conclusion that a mechanism which can be blocked by soluble tumor membrane and which is not dependent on an intact thymus-derived response, may be responsible for the active elimination of small tumor inocula.

CAN SUPPRESSOR CELLS MODULATE THE ANTITUMOR EFFECTOR MECHANISM ?

Although we had demonstrated that T depleted mice have an intact effector mechanism for small tumor inocula, it was not clear whether suppressor cells might still be able to modulate the rejection. An aliquot of 3.5×10^7 suppressor cells was adoptively transferred to non-immune A/J mice at the same time as a s.c. challenge with 10^2 to 10^5 Sa1509a cells. As illustrated in Figure 1a, there is no discernible effect on the frequency of tumors or on the growth rate in the primary host. This contrasts with the enhanced tumor growth seen when suppressor cells were transferred with a 10^6 tumor cell inoculum into immune animals (Fig. 1b).

DO SMALL INOCULA GENERATE SUPPRESSOR T CELLS ?

Reports by Mengerson et al (6) indicate that the administration of repeated small doses of tumor can increase the susceptibility of syngeneic mice to a subsequent challenge with a large tumor inoculum. Even though we were unable to demonstrate an enhanced mortality rate on secondary challenge after a single small tumor inoculum (2) it was considered valuable to determine whether these small inocula can generate suppressor T cells. We obtained thymocytes from animals seven to ten days following the injection of 10^2 to 10^3 Sa1509a cells s.c., intravenously (i.v.), or intraperitoneally (i.p.). The cells were then adoptively transferred into immune A/J mice, followed immediately by a s.c. injection of 10^6 Sa1509a cells. Thymocytes taken from animals receiving small tumor inocula s.c. or i.v. were not able to generate suppressor cells in contrast to thymocytes taken from mice which

received 10^6 cells s.c. (Fig. 2a). Interestingly, thymocytes obtained after small i.p. inocula, the only route of administration by which small inocula will consistently grow (2), enhanced tumor growth rate, especially noticeable on day 5. (Figure 2b).

DOES THE IMMUNE RESPONSE ENHANCE TUMOR TAKE ?

If the appearance of morphologically detectable tumor is always associated with induction of effector cells and suppressor cells, could the immune response stimulate the growth of tumor, as suggested by Prehn (7). To test whether an ongoing immune response to the tumor could enhance the tumor frequency of small inocula we examined the following model. A 10^6 cell inoculum of the non-metastisizing Sa1509a was implanted on one side of the back of a non-immune host along with a 10^2 or 10^3 cell inoculum on the opposite side. After observing the animals for over 30 days we were unable to detect any increase in the tumor frequency (Table II).

In a second experiment DBA/2 mice received bilateral heel injections of 10^7 L5178Y tumor emulsified in complete Freund's adjuvant 14 days prior to challenge with a small tumor inoculum. Again, no increase in tumor frequency was observed (Table III).

DOES THE AGE OF THE HOST EFFECT ITS ABILITY TO ELIMINATE TUMOR ?

The immune surveillance hypothesis, as developed by Burnet (8) from earlier suggestions by Thomas (9), postulates that the thymus dependent adaptive immune system provides the primary defence against neo-

plastic clones. Burnet suggested that the high incidence of tumors late in life may be a result of the degeneration of the immune response with aging (10). To test this hypothesis we examined the ability of moderately old (> 44 weeks) or young (6 to 8 weeks) mice to eliminate tumor inocula of all sizes. It was clearly established for both the L5178Y and Sal509a that there is no increase in tumor frequency or alteration in tumor growth rate in relatively older mice (Table V and Fig. 3). We conclude that the effector mechanisms responsible for the elimination of tumor are not affected by the age of the host.

DISCUSSION

The elimination of small tumor inocula can be blocked by the administration of solubilized tumor membranes resulting in a significant increase in tumor frequency. The effector mechanism, however, cannot be influenced by T-cell ablation (2), suggesting that it is thymus independent. Furthermore, we have established that animals which had rejected these small inocula had no memory of the primary challenge on re-exposure to the same tumor. Secondary challenge with small tumor inocula did not result in a reduced frequency of tumors, and rechallenge with large inocula exhibited the same latency, growth rates and survival rate as animals which had not been exposed to small tumor inocula (2). From these observations, we concluded that the characteristic immunological memory of the thymus dependent immune response was not stimulated by a small tumor dose.

In the present series of experiments, we have further characterized

the mechanism responsible for the elimination of small tumor inocula. It is apparent that suppressor T cells are not able to modulate the primary responses to small or large tumor inocula, in contrast to their effects on the secondary response in immune mice (4). The inability of suppressor T cells to improve the frequency of tumors produced by small inocula indicates the presence of a different effector mechanism from the secondary host. However, the inability of suppressor T cells to influence the growth rate of large tumor inocula in the primary host may only reflect the fact that once tumors are established they are able to inhibit the immune response so efficiently that the addition of suppressor cells has no effect.

The observation that both large progressively growing tumors as well as small intraperitoneal tumor inocula generate suppressor cells, contrasts sharply with the failure of small s.c. and i.v. tumor inocula. The latter route of administration produces tumors in only a small proportion of mice, while large inocula and intraperitoneal injection always results in tumor growth. Progressive growth of a tumor therefore may be a prerequisite for the production of suppressor cells. The additional observation that effector cell memory is seen only after surgical ablation of actively growing tumors and never in mice which had rejected small tumor inocula, suggests that induction of memory also occurs only with progressive tumor growth.

Finally, we have shown that neither the growth rate or tumor frequency of small and large tumor inocula is directly affected by the age of the host. This implies that the components of the immune response which

have been shown to deteriorate with aging (11) do not reduce the capacity of the host to affect tumor growth or rejection. With this observation one must also consider the possibility that the typical age distribution of tumors (10) is not a result of deterioration of the hosts immune resistance to random oncogenic mutations, but may reflect the non-random induction of tumors by oncogenic agents with long latency periods (12), or an increased susceptibility of aging cells to chemical carcinogens (13). The non-adaptive surveillance (NAS) mechanism then, may reduce the frequency at which mutations result in tumors equally at all ages and the increased tumor incidence in old age reflects the high mutation rate at this time of life. The mechanism by which oncogenic mutations may escape from surveillance is suggested by the observation that soluble tumor antigen can block the rejection of small tumor inocula.

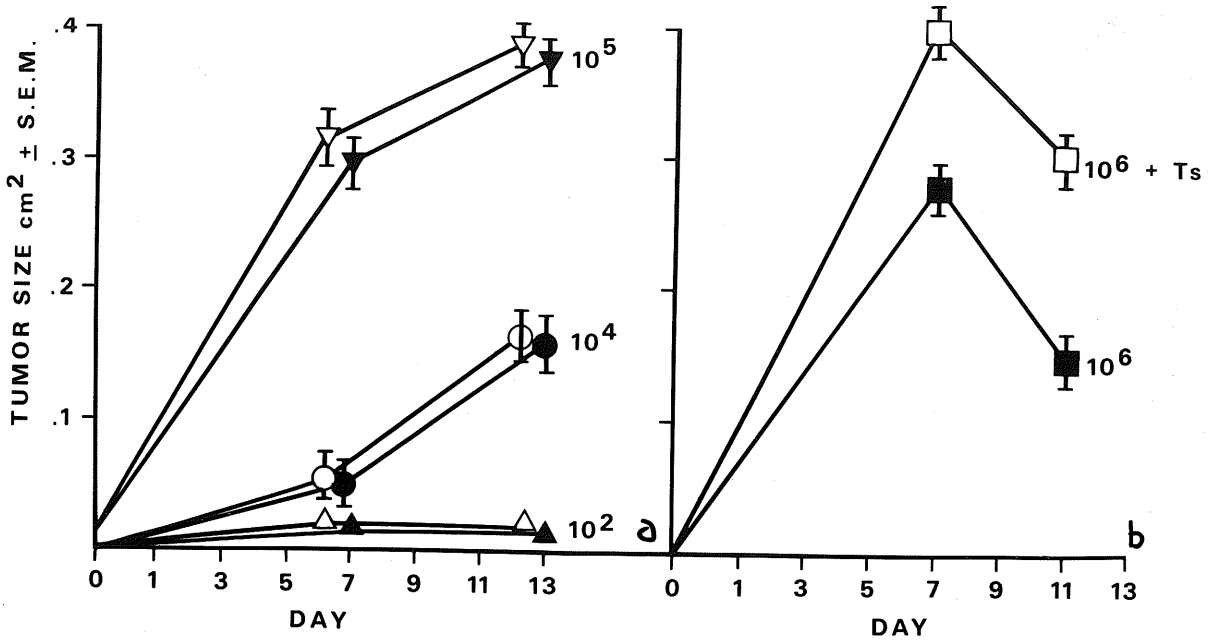

Figure 1: The effect of suppressor cells on the primary and secondary response to syngeneic tumor. (A) Non immune A/J mice were subcutaneously challenged with 10^2 (△ , ▲), 10^4 (○ , ●), and 10^5 (▽ , ▼) Sal509a. Open figures represent mice which received 3.5×10^7 suppressor cells intravenously while closed figures are mice which received no suppressor cells. (B) Immune A/J mice injected with 10^6 Sal509a subcutaneously and 3.5×10^7 suppressor cells intravenously (□) are compared to immune A/J mice receiving only 10^6 Sal509a (■). A/J mice were made immune by surgical ablation of a subcutaneous tumor.

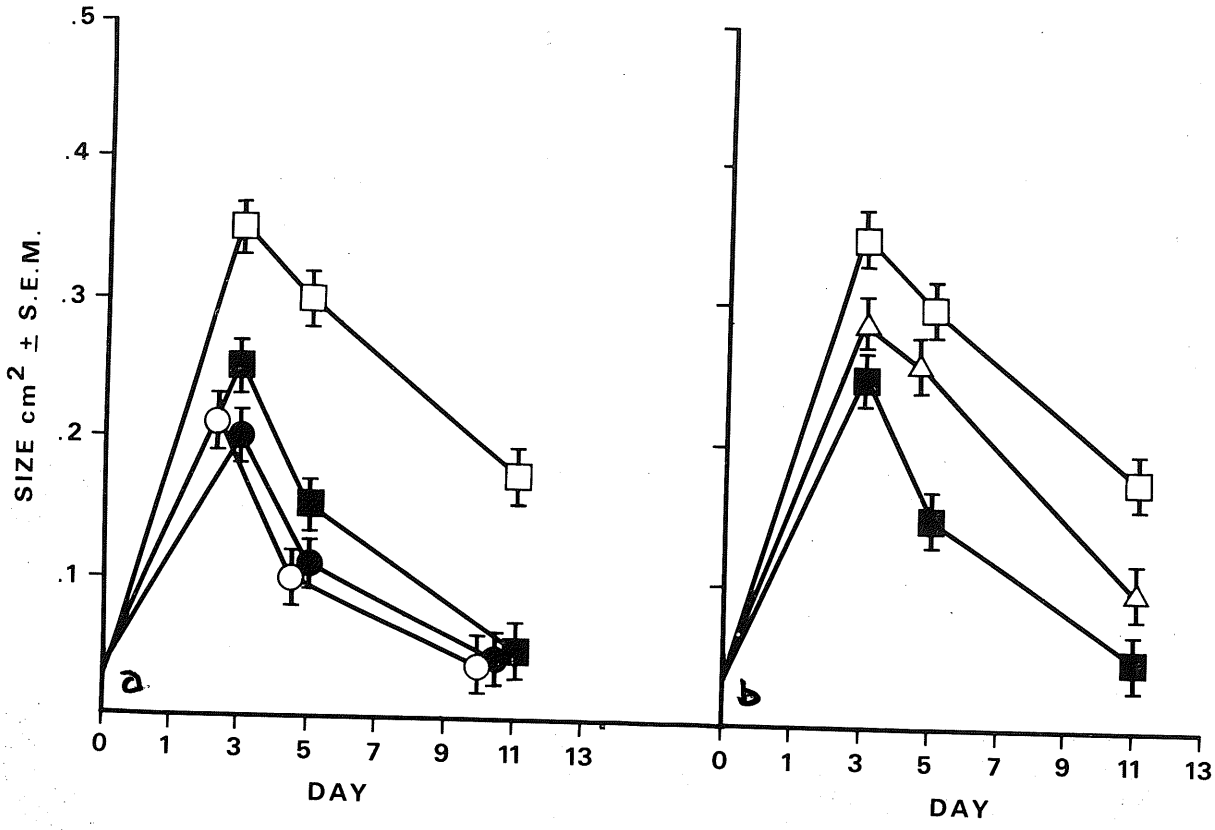

Figure 2: Induction of suppressor cells by small tumor inocula. (A) Suppressor cells were obtained from thymus 7 days after the injection of 10^2 Sal509a subcutaneously (○), 10^2 intravenously (●), and 10^6 subcutaneously (□). The cells were then intravenously injected into immune A/J recipients and the effect on the growth of a subcutaneous injection of 10^6 Sal509a was compared to mice receiving no suppressor cells (▣) (B) Suppressor cells obtained from thymus 7 days after the injection of 10^2 Sal509a intraperitoneally (△) and 10^6 subcutaneously (□) are compared to untreated controls (▣) as outlined in (A).

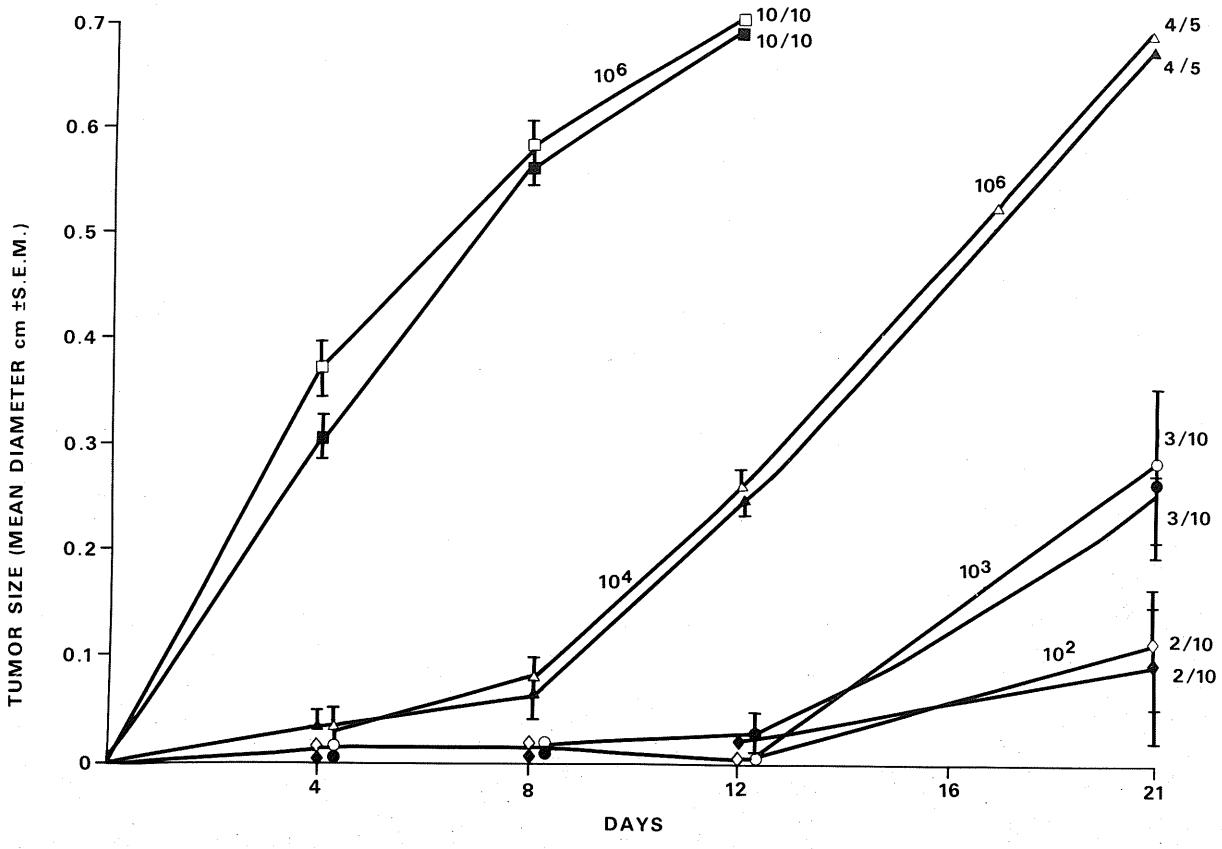

Figure 3: The effect of age on the growth rate of Sal509a. Young A/J mice (6-8 weeks)(closed figures) and old A/J mice (44-52 weeks)(open figures) were subcutaneously injected with 10^2 Sal509a (◇, ◆), 10^3(○, ●), 10^4(△, ▲) and 10^6(□, ■) and the growth rate of the tumor observed for 21 days.

TABLE 1

The Rejection of Small Tumor Inocula in Normal, Antigen Treated and Immunodeffcient Syngeneic Mice

Group	Treatment	Strain	Tumor	Tumor dose[4] (viable cells)	Tumor frequency[5] Number	Percent
1.	--	A/J	Sa1509a	1×10^2	3/20	15
2.	1.0 μg soluble[1] Sa1509a membrane i.v.	"	"	1×10^2	7/10	70
3.	--	"	"	1×10^3	5/20	25
4.	Adult thymectomy lethal irradiation bone-marrow reconstitution[2]	"	"	1×10^3	2/5	40
5.	--	"	"	1×10^6	20/20	100
6.	--	DBA/2	L5178Y	0.5×10^2	3/15	20
7.	1.0 μg soluble[3] L5178Y membrane i.v.	"	"	0.5×10^2	12/23	52
8.	Adult thymectomy lethal irradiation bone-marrow reconstitution[2]	"	"	0.5×10^2	0/15	0
9.	--	"	"	10^5	15/15	100

(1) 3M KCl extract of Sa1509a was prepared by the method of Forbes et al (14).

(2) Mice were irradiated and bone marrow reconstituted 4 weeks after thymectomy.

(3) Papain digest of L5178Y membrane was prepared by the method of Maramatsu et al (15).

(4) Subcutaneous mid back inoculation.

(5) Tumor frequency was assessed 25 days after implantation of tumor. No new tumors appeared after this time.

TABLE II

The Effect of a Simultaneous Injection of a Large Tumor Inoculum on the Frequency of Tumors Produced by a Small Tumor Inoculum

	Tumor inocula			Tumor Frequency *			
				Left Side		Right Side	
Group	Left Side	Right Side	Tumor	No.	%	No.	%
I	10^2	--	Sal509a	2/5	40	--	--
II	10^2	10^6	Sal509a	2/5	40	5/5	100
III	--	10^6	Sal509a	--	--	5/5	100
IV	10^3	--	Sal509a	3/5	60	--	--
V	10^3	10^6	Sal509a	3/5	60	5/5	100
VI	--	10^6	Sal509a	--	--	5/5	100

* The experiments have been performed with an additional 10 mice/group with similar results. The tumor growth rate on day of appearance of tumor was not remarkably different in any group.

TABLE III

Incidence of Tumors Following Pre-Immunization

With L5178Y in Complete Freund's Adjuvant

Treatment	Tumor Inoculum	Tumor Incidence	
		Number	Percent
10^7 L5178Y in CFA*	5 x 10^1 L5178Y	5/15	33
Medium	5 x 10^1 L5178Y	6/15	40

*bilateral heel injection 14 days prior to tumor challenge

TABLE IV

The Effect of Age on the Frequency of Tumors

Tumor Inoculum	Strain	Tumor Incidence[1] < 8 weeks No.	%	> 44 weeks No.	%
10^2 1509a	A/J	1/5	20	1/5	20
10^3 "	"	1/5	20	2/5	40
10^4 "	"	3/5	60	3/5	60
10^5 "	"	5/5	100	5/5	100
10^6 "	"	5/5	100	-	-
10^1 L5178Y	DBA/2	1/10	10	1/10	10
$5x10^1$ "	"	2/8	25	2/10	20
10^2 "	"	4/10	40	3/10	30
10^3 "	"	6/10	60	5/9	55
10^6 "	"	5/5	100	--	--

[1]Observations were made at 30 days after tumor inoculation. No further tumors appeared after this time.

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CHAPTER 4 and 5

The contribution of Dr. A. Greenberg involve those systems dealing with data other than the S1509a tumor results.

XI.

DISCUSSION

The demonstration that immunosuppressor T cells can negatively modulate the immune cytolytic effector response in mice has been documented in the preceding chapters. The scope of the present work has focused upon three major problems of the host-tumor relationship and these were (i) the isolation and characterizations of IST (ii) the definition of the subcellular component. ISF and its possible locus of action on the effector limb of the immune response and (iii) the different parameters of the tumor-host relationship which are operative in the primary tumor-bearing host carrying a small tumor load.

The studies have made use of a methylchloranthrene-induced fibrosarcoma S1509a syngeneic to A/J (H-2^a). The demonstration that many such carcinogen-induced tumors possess tumor specific antigens which are immunogenic was extended to the work described herein. Utilizing A/J mice which had been rendered immune to S1509a, it was found that adoptive transfer of thymus-derived cells obtained from animals bearing a progressively growing tumor into immune A/J significantly inhibited their immune capacity to reject a subsequent tumor challenge.

The cells endowed with this suppressive capacity were cortisone resistant, implying that they were ontogenically mature. Furthermore, the regulatory cells arise within 36 hrs of tumor exposure and disappear within 5 days of tumor removal indicating that this subpopulation of T cells arises relatively quickly but is short-lived after antigen removal.

Suppressor cells appear in a variety of organs including the bone marrow, lymph nodes, spleen and thymus. Interestingly, splenectomy of primary tumor-bearing animals led to a temporary arrest of tumor

growth, whereas sham splenectomy had no such effect indicating one could selectively deplete a pool of regulatory cells.

It was found that suppressor cells were light in density and could be physically separated from other lymphoid cells with the use of Ficoll gradients. Although not described in Chapter II, such a cell population, when examined under light microscopy, contained many cells with prominent nucleoli. This observation by itself would imply a relatively immature cell, but taken together with the observed cortisone resistance might indicate a transition cell. However, it is uncertain at this point the precise time in ontogeny at which suppressor cells arise.

The suppressor cells were found to operate via suppressor factors which were obtained from the ruptured cell extracts. In Chapter III, extensive immunochemical characterization has shown suppressor factors to be protein, labile at either pH 2.8, or on exposure to temperatures of 56°C for 1 hr. Interestingly, such factors were found to be antigen specific, and to possess determinants coded by genes existing in the K end of the H-2 major histocompatibility complex. Such tumor suppressor factors conceivably reflect a parallel control mechanism of CMI as seen with other suppressor products operative to limit allotype specific (307) or carrier-specific (308) antibody responses.

The primary response to tumor was next explored to elucidate the nature of the effector cells responsible for the elimination of minimal inocula and to define the regulatory mechanisms which limit that response. Using the same tumor S1509a but at doses of $< 10^4$ cells,

it was observed that effector cells responding to tumor were not T-cell dependent. Furthermore, such primary or natural killer cells were not amenable to suppressor cell influence but were able to be inhibited by S1509a soluble tumor extracts. Therefore, I have shown that the regulation of effector mechanisms apparently differs in the primary and secondary response. A comprehensive analysis of all aspects of regulation attempting to integrate all the data as it applies to the S1509a tumor, starting at the time of a small primary inocula and extending until the tumor is large and rapidly growing will now be attempted.

From the data reported in Chapter IV and V, it is clear that a small S1509a tumor inocula $< 10^3$ cells is recognized by a non-T cell effector mechanism. The identity of the cells or agents responsible for small tumor elimination is unclear, but candidates may be natural antibody, or natural killer cells (306). Interestingly, such a primary effector mechanism is not apparent in the peritoneal cavity, as small inocula grew consistently in that area. It would appear that this 'surveillance' mechanism may be T-cell independent as T-cell deprived animals, so-called B mice, did not demonstrate an increased incidence of tumor takes after small tumor inocula. Further attempts to influence the number of tumor takes by adoptive transfer of exogenous IST obtained from animals challenged with a large tumor inocula (10^6) were consistently futile. This contrasted sharply with the marked increase of tumor takes (20% to 75%) which resulted when 3MKCl solubilized tumor membranes were administered passively at the time of the initial small inocula. It was further found that small tumor inocula would

not stimulate the development of suppressor cells, unless the inocula escaped elimination. In this case, suppressor cells become evident. The conclusions are that (1) primary small tumors are not eliminated entirely by T-cell dependent mechanisms; (2) exogenously produced suppressor cells on transfer cannot influence the take of tumors; and, (3) that soluble tumor membrane products effectively block primary tumor elimination.

At a later stage of tumor growth comparable to the situation when 10^6 cells are administered, suppressor cells appear to be rapidly generated, possibly as a result of continuous release of tumor antigen. These IST are apparent in the bone marrow, lymph nodes, spleen and thymus of the host. Suppressor cells may then act in a tumor antigen specific manner to either arrest differentiation of effector cells or alternately to directly limit the functional capacity of effector cells. This is seen, in particular, when such IST are administered to immune animals which are engaged in the rejection of a tumor transplanted five days previously . The transferred IST leads to a remarkable 'escape' of tumor rejection, probably by one of the mechanisms alluded to.

IST function via ISF which is either passively carried on the cell surface or is in fact elaborated by the IST cell itself. The ISF operative in this system displays tumor antigen specificity and carries antigens of the H-2 complex and, in particular, those of the K-I region of the $H\text{-}2^a$ mouse haplotypes. (Please see pages 30-35 for discussion of the H-2 complex).

This identification of the determinants possessed by ISF was also described in studies dealing with the radiolabeling by I^{125} of ISF which are reported in the appendix. By this procedure, independent confirmation of the identity of ISF was accomplished establishing that ISF was an H-2 linked or produced cell membrane product. Having described the antigen specificity of ISF, and the action of IST on tumor rejection, I will now attempt to formulate the following model of action for ISF. IST are generated by tumor antigen of S1509a. These IST pick up or produce a tumor-specific binding factor, ISF. These factors are then liberated as a soluble membrane protein from the cell surface after the ISF has interacted with specific tumor antigen. The ISF-antigen complex can now, by virtue of the lymphocyte cytophylicity of ISF itself (data not shown), bind to effector cells or their precursors. This interaction probably is sufficient to render such cells temporarily inactive. The cytolytic effector response is, therefore, diminished and tumor growth continues relatively unabated.

This scheme of a biologically active subcellular component is analogous to the recent observations by Munro *et al.* (237), Tada (222), Ramseier (256) and de Weck (257) who all have demonstrated that certain cell-derived factors which carry determinants coded by the MHC can have potent enhancing or suppressive effects. Despite the antigen specificity of ISF and other such suppressive factors, there are many reported factors that have not been fully scrutinized for their relationship to the MHC (241-246, 253-255), and many entirely non-specific factors also operational in a variety of immune responses

(40,221,225,243). The role of these non-specific factors (241-243) in the presently studied system is not apparent, but possibly may be significant.

At this point it is impossible to reconcile the extensive data of H-2 linked suppressive or amplifier factors with the equally extensive data concerning the observations that T cells might interact with antigen via idiotype-like receptors. One resolution, which could account for both products playing a biological role, would be that H-2 coded products represent the light chain analogue of ordinary immunoglobulin κ or λ chain, and as such contribute a degree of the binding energy for antigen on the T cell. The remainder of the binding site would be derived from the gene products of the heavy chain variable region (V_H) (134). This notion will remain highly speculative until such time as the T-cell receptor can be identified and characterized.

The ISF complex as envisioned in the proposed scheme is composed of a T cell product possessing H-2 determinants and tumor antigen and is very efficient in negatively modulating the response to tumor in immune A/Jax mice. Perhaps the action of similar complexes might be offered as an explanation for the H-2 influence seen for tumor susceptibility as shown by Lilly *et al.* (252) (Please see pages 31-33 for H-2 and neoplastic susceptibility), or, alternately, tumor resistance (259), inasmuch as comparable biological mediators to ISF may be present. It would be more probable, however, that tumor resistance or susceptibility is polygenically influenced.

As final consideration, it is conceivable that the observations reported herein might be used to devise immunotherapies for human malignancies. One major advance would be the production of antisera to suppressor factors operational in tumor systems, as was shown to be effective when anti-ISF (Chapter III and appendix) was used to functionally inactivate IST in the tumor system studied. It is also possible that surgical extirpation of the spleen might aid tumor therapy as this would deplete a significant suppressor pool (264). Other potential applications of the experimental system described are the reduction of ISF protein production by the use of specific chemotherapy.

The ultimate goal in the therapy of neoplastic disease is the elimination of malignant cancerous cells without generalized damage to normal cells. Potentially immunological recognition can discriminate between the antigens of tumor cells and those of normal cells. It is of some importance to define and eliminate suppressive mechanisms which prevent cytolytic effector responses from eliminating tumor cells.

ORIGINALITY OF WORK

The theme that a thymus-derived (T) cell can limit the effector cytolytic response to tumor provides an explanation for the observed failure of tumor rejection in many situations. Prior to the work done in this thesis, this notion to explain such failures of the immunological response to a progressively growing tumor was not enunciated.

Furthermore, at a very early period of tumor development, I have demonstrated that a different effector mechanism was responsible for tumor cytolysis. This mechanism, as opposed to secondary responses to tumor, was found to be blocked by soluble tumor antigen.

The original contributions of this work relate 1) to the characterization and functional description of suppressor cells and factors and 2) the description of the primary natural effector process and certain of its regulatory mechanisms.

APPENDIX

The experiments described in this section deal with independent confirmation of the identity of ISF using a cell-surface radiolabeling technique. They have been separated from the body of experimental work as they have been done to establish the immunochemical nature of ISF *in vitro* and do not relate to function of ISF *in vivo*.

Lactoperoxidase Labeling

For the purpose of lactoperoxidase labeling of the cells, the method of Marchalonis (35) was used as modified by Vitetta and Uhr. Briefly, aliquots of thymic cells obtained from the Ficoll density discontinuous gradient were iodinated with 400 μCi of I^{125} (New England Nuclear Corp., Boston, Mass.) per 2 x 10^7 cells, with the use of lactoperoxidase, B grade (33 μm) (Cal. Biochem., San Diego, Calif.). Lactoperoxidase, at a concentration of 6 μg/ml was used. A constant carrier iodide concentration of 10 μ M KI was used to allow the enzyme to function in the most efficient substrate range. H_2O_2 was used at a concentration of 44 μM to ensure maximum incorporation and activation of the enzyme. Earle's Balanced Salt solution without phenol red was adjusted to pH 7.2 by using 5.6% $NaHCO_3$. The stock solution was frozen and thawed ($E_{\mu M}$ at 412 = 114). Most operations were done with the cells cooled on cracked ice and the labeling was done at 25°. Cells were washed 2-4 times in the balanced salt solution containing 10 μM KI and adjusted to a density of 2 x 10^7 cells/ml. Lactoperoxidase was added from the stock solution to a final concentration of .33 μM. After addition of 400 μCi x I^{125}/ml, the reaction was begun by the addition of 0.03% H_2O_2 to the cell mixture. After a 1 minute period a second addition of H_2O_2 was made. One minute later, the cells were washed free of enzyme and unreacted radiolabeled I^{125} was removed using a discontinuous BSA gradient, followed by several washes in chilled Earle's solution. Subsequent to this, the cells were subjected

to snap-freezing and thawing followed by ultracentrifugation at 100,000 g for 1 hour. Following, the labeled soluble factor was dialyzed against BBS for 2 days, recentrifuged and counted in a well type scintillation counter (Nuclear Chicago).

For the second part of this experiment, precipitation by antisera was used to specifically isolate the various factors. As first antisera, a battery of sera were used including BALB/c-anti-A/J, C3H/HeJ-anti A/J, Anti-Fr_3, B10.D2-anti B10Br, NMS, RWS, R anti M $F(ab')_2$, R-anti-μ. To precipitate these antisera, the appropriate dilution of rabbit-anti mouse $F(ab')_2$ or sheep-anti rabbit globulin was used, after the amount and ratio needed for maximal precipitation was determined. Separate aliquots of labeled soluble factor were precipitated with different antisera, in the cold for 24 hours, with constant rotation. After this, the tubes were spun down at 1200 RPM for 6 minutes and the supernatant removed and saved. The precipitates were counted after several washes and the ratio to specific controls were calculated as well as the absolute cpm of the precipitate. The supernatant that had been saved was then treated again with different antisera and the process repeated for another 24 hours. In this manner, if the different antisera were directed at the same specific product, one could predict that on the second part of the experiment, one would not expect any further precipitation of radiolabeled product. The results of this work are reported in the following experiment.

The Ability to Detect Suppressor Factor on the Cell Surface by the Lactoperoxidase Catalyzed Incorporation of I^{125}

The ability to detect surface moieties on lymphocytes by the lactoperoxidase catalyzed incorporation of I^{125} was reported by Marchalonis (35). Vitetta and Uhr (249) have used the technique to define surface Ig and as well as I region and H-2 products, on T and B cell surfaces. By using a similar technique as Vitetta (249) the following experiments were performed. The following antiseras were used anti-ISF. (Group I), anti-IS.F. which had been absorbed with 10^8 C3H/HeJ lymph node cells (Group 2). Anti-ISF which had been absorbed with 10^8 BALB/c J lymph node cells (Group 3), control rabbit whole sera (decomplemented)(Group 4). C3H/HeJ anti-A/J (Group 5), BALB/c J anti-A/J (Group 6)-$B10D_2$ anti-B10Br (Group 7) control-mouse whole sera (decomplemented) (Group 8). The antisera used to precipitate these above antisera were sheep anti-rabbit globulin when the first sera was produced in rabbit, or alternately rabbit anti-mouse $F(ab')_2$ to precipitate antisera that had been raised in mice. It was shown that anti-IS.F. could specifically precipitate out a labeled surface moiety (Group I). Moreover, BALB/c anti-A/J (anti-H-2K-I^k antisera) used on the supernatant of the anti-IS.F. treated TBH thymus extract could not precipitate any label over and above that of control (Group 9). It is of interest to note that there appears to be some degree of cross reactivity between some H-2D end and H-2K end structures as the experimental group 5 vs. 13 or 6 vs. 14 clearly demonstrate. That is to say, when the supernatant of the BALB/c J anti-A/J (anti-H-2K-I^k)

(Group 6) or the supernatant from the C3H/HeJ anti-A/J (anti-H-$2D^d$) (Group 5) precipitation was further tested with anti-ISF, (Group 13-14) respectively a similar amount of I^{125} label was precipitated. This suggests some cross-reactivity between certain D and K region structures. Shreffler (262) has recently demonstrated such cross-reactivity, and these results are not unlike the reported biochemical data of Nathenson on cross-reactivity (240). It is most apparent that absorption of the anti-ISF with C3H/HeJ lymph node, (Group 2) cells prior to use in these experiments was sufficient to deplete the antisera of any ability to precipitate label. Furthermore, when the supernatant was then tested with BALB/cJ anti-A/J anti-H-$2K^k$-I^k, there was a definite increase of label precipitate when compared to control, reinforcing that the identity of the product recognized by anti-ISF was a product of the K^k end of the H-2 complex. The use of specific antisera directed at only the H-2 (B10.D2 anti-B10.Br) (Group 10) demonstrate that the specific entities recognized by anti-ISF were limited to certain products of the MHC. The anti-ISF could not precipitate any further label from the supernatant remaining after B10.D2 anti-B10.Br treatment. If this data is taken along with the observation that incubation of anti-IS.F with C3H/HeJ (K haplotype) lymph node cells resulted in the loss of that antisera's capacity to precipitate a surface product, then it follows that K or I region products are cross-reactive to or are linked to the surface product recognized by anti-IS.F. antisera. These observations as seen from the data of this representative experiment report no large differences as compared to control. However, these differences have been found consistently and these experiments have been repeated (3X). It should be noted that the

ratio differences are usually in the range of 1.5 - 2 x control and this is not dissimilar from the results of others (Greenberg-personal communication).

Therefore, the demonstration that H-2^k haplotype lymph node cells deplete the antisera directed at the suppressor factor, i.e., anti-ISF, of any detectable activity, strongly suggests that the moeity recognized by the anti-ISF was likely a K^k end product and in particular that it is, in fact, either K or I region products or linked to these products. Furthermore, in experiments done but not reported, the anti-ISF failed to precipitate any labeled surface product of 1509a tumor cells over that of control. Whereas the antisera to the K end or D end did precipitate some label. This fact suggests that the anti-ISF recognized some moiety that was only part of the spectrum of moieties recognized by the anti-H-$2K^k$ end or H-$2D^d$ end antisera. It further suggests that the labeled surface product is not a tumor cell product but rather represents some unique lymphocyte surface antigen. In other experiments done but not reported, the ability of the suppressor factor to be precipitated by anti-Fab or anti-μ was assessed. In every experiment there was no discernible cross-reactivity between anti-Fab or anti-μ and anti-ISF. This corroborates the *in vivo* experiment conducted after passage of the factor through reverse immunosorbents of anti-$F(ab)^2$. The evidence that is presented suggests, therefore, some aspect of identity or linkage with some K-I region antigen, of the factor that is recognized by anti-ISF.

Anti-ISF treatment *in vitro* has also been shown to deplete thymus cells or TBH of suppressive activity and is presumably in this case interacting with the ISF. These experiments done *in vitro* tend

to corroborate the notion that IST function via ISF which is a cell membrane product coded by or linked to products of the H-2 complex.

Table I

ANTI-ISF PRECIPITATES AN I^{125}-LABELED MEMBRANE PRODUCT OF TUMOR-BEARING ANIMAL THYMOCYTE

GROUP	I^{125} THYMUS CELL FACTOR(S) ANTISERA USED TO PRECIPITATE FACTORS		MEAN CPM x 10^3 PRECIPITATED	RATIO Specific Control
	1st Antisera	2nd Antisera		
1.	Anti-ISF	Sheep anti-R globulins	5.1	2.2
2.	Anti-ISF absorbed with C3H lymph node cells	"	3.1	1.3
3.	Anti-ISF absorbed with BALB/c lymph node cells	"	5.4	2.3
4.	Rabbit whole sera	"	2.4	1.0
5.	C3H/HeJ anti-A/J	R anti-M F(ab')	3.9	1.6
6.	BALB/c anti-A/J	"	3.4	1.4
7.	B10D.2 anti-B10.BR	"	4.6	1.9
8.	Mouse whole sera	"	2.4	1.0

Table I. (continued)

GROUP	SUPERNATANTS OF ANTISERA		MEAN CPM x 10^3	RATIO Specific Control
	3rd	4th		
9.	Supernat. from 1. BALBc/J anti-A/J antisera	R anti-MFab	1.5	1
10.	Supernat. from 2. BALBc/J anti-A/J antisera	"	2.1	1.32*
11.	Supernat. from 3. BALBc/J anti-A/J antisera	"	1.7	1.0
12.	Supernat. from 4. Mouse whole sera	"	1.6	1.0
13.	Supernat. from 5. anti-ISF antisera	Sheep anti-R GL	1.5	1.0
14.	Supernat. from 6. anti-ISF antisera	"	1.5	1.0
15.	Supernat. from 7. anti-ISF antisera	"	1.5	1.0
16.	Supernat. from 8. Rabbit whole sera	"	1.7	1.0

* This result was found consistently in a number of experiments (3) and is regarded as reflecting meaningful differences ($p < .025$) (Student's T-test).

XI.

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BIBLIOGRAPHY

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