CELLULAR RECOGNITION OF TIMOTHY POLLEN ANTIGENS

by

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INTRODUCTION

Statement of Problem:

A low molecular weight, non-precipitating antigen from timothy pollen extract, Antigen D (AgD), is monovalent for the major antigen which induces reaginic antibody in humans. Antigen D inhibits specific reagin activity in vitro and appears to suppresses its formation when administered in alginate to allergic patients. An understanding of the molecular composition of AgD, i.e. hapten and carrier determinants, and of how it suppresses at a cellular level, would provide insight into how reagin production is regulated, and would also provide a basis for defining and isolating suppressive fractions from other allergens. As a consequence, therapy of allergic disease might be improved.

Small fragments have been isolated from other allergens, and in the case of penicillin allergy, hapten therapy has been beneficial. The timothy pollen system is a potentially good model for studying hapten suppression because several timothy pollen antigens have been well defined physicochemically and antigenically.

I chose to evaluate suppression of allergic antibody formation in mice rather than humans because their lymphocyte populations are better defined and can be more effectively isolated. My first goal was to induce IgE production in mice and to determine optimum conditions for obtaining a reproducibly high and boosterable titer of homocytotropic antibody. It was also necessary to define the speci-

ficity of the antibody produced. If mice did not recognize the antigen shared by AgD, then AgD suppression and inhibition experiments would be meaningless.

Two approaches were taken to study the suppressive effects of AgD. First, AgD was administered in vivo to try and suppress an ongoing IgE response, and second, the effects of AgD on lymphocyte responses in vitro were analyzed. Because AgD proved to be immunogenic in mice, in vivo studies were discontinued.

In order to study antigen-specific reactivity at a cellular level, I decided to evaluate an in vitro lymphocyte transformation system to determine whether it could be used to measure B and T cell proliferation induced by purified pollen antigens. Using this system I hoped to be able to distinguish differences in response patterns using lymphocytes from mice immunized in different ways to favor IgE or IgG synthesis; comparing dose response curves and AgD inhibition profiles might allow distinction between T and B cells involved in IgE and/or IgG production.

I also wanted to use purified B and T lymphocyte populations to define hapten and carrier specific recognition events, and then to test the inhibitory effect of AgD on these. With this system I could determine the minimum number of distinct cell populations responding to different determinants of timothy pollen. Furthermore, I could determine whether AgD has both hapten and carrier determinants capable of causing inhibition, and from this information I could predict the cellular site or sites of AgD suppression in vivo.

Timothy Pollen Antigens:

The isolation and immunological characterization of the two major antigens of timothy pollen, Antigen A (AgA) and Antigen B (AgB), were described by Malley and Harris (1967). The two antigens are separable from one another on DEAE-Cellulose, have molecular weights of 13,000 and 10,500 daltons respectively, and have distinct antigenic determinants. Immunodiffusion analysis with hyperimmune guinea pig serum against the crude pollen extract (WST) demonstrated that the major precipitin line against AgA showed nonidentity with the major precipitin band against AgB; however, AgA also had a small amount of antigenic material which was identical with AgB.

The low molecular weight component of timothy pollen, Antigen D (AgD), was isolated from the crude pollen dialysate by gel filtration (Malley, Campbell, and Heimlich, 1962). The molecular weight of AgD was estimated from its elution volume to be 5,000 daltons. Antigen D consists of 53 amino acid residues rich in tryptophan and proline, cellobiose, and a flavonoid pigment, quercitin (Malley, Saha, and Campbell, 1964). It was determined that AgD has the same antigenic determinant as AgB although in monovalent form (Malley and Harris, 1967). Antigen D will not precipitate with hyperimmune serum but will inhibit precipitation with AgB. Furthermore, AgD will inhibit homocytotropic and heterocytotropic antibodies against AgB; it will inhibit passive cutaneous anaphylaxis (PCA) in guinea pigs, Prausnitz-Kustner (P-K) reactions in man, and allergic histamine release from monkey lung tissue passively sensitized with human anti-

timothy serum or from leukocytes of allergic individuals.

Treatment of Timothy Pollen Allergy:

Because AgD is monovalent for the AgB determinant which induces most timothy specific reagin in humans, and because it is only weakly immunogenic (Malley and Perlman, 1969), it was felt that AgD might be useful for hyposensitization therapy of allergic individuals. Results of initial clinical trials (Malley and Perlman, 1970) indicated that AgD therapy caused greater decreases in clinical symptoms and circulating reagin titers than could be obtained with WST therapy. Antigen D also resulted in decreased basophil reactivity to antigen challenge in vitro and decreased lymphocyte transformation of peripheral blood leukocytes challenged with antigen. Furthermore, proliferative responses of lymphocytes from untreated individuals could be inhibited by preincubation with AgD; AgD also suppressed the formation of MIF and blastogenic factor (Malley, Wilson, Barnett, and Perlman, 1971; Malley, Crossley, Baecher, Wilson, Perlman, and Burger, 1973). These observations suggested that AgD was neutralizing circulating reagin, reacting with cell bound reagin and inhibiting antigen-induced histamine release, and also suppressing antibody formation.

Circulating antibody and B cell receptors have the same specificities; therefore, AgD could inhibit B cells directly. Moreover, lymphocytes releasing mediators are T cells, and since AgD inhibits this function, it could presumably interact with T cells as well.

To determine which lymphocyte population is suppressed by AgD, I developed a mouse system where T (thymus-derived) and B (bone marrow-derived or bursal equivalent) lymphocytes are well defined and separable on the basis of distinct surface markers (reviewed by Miller, Basten, Sprent, and Cheers, 1971); T lymphocytes have a theta antigen and B lymphocytes have surface immunoglobulin and receptors for the third component of complement and the Fc portion of immunoglobulin. The murine system was also desirable because the mouse homocytotropic antibody response has been extensively studied in the last few years. Cell interactions necessary for antibody production have been well defined. Since I will be discussing hapten and carrier recognition events of B and T lymphocytes respectively in the timothy pollen system, an understanding of cell interactions is important for evaluating both in vivo antibody production and in vitro responses of lymphocytes to antigen.

Mouse Homocytotropic Antibodies:

Two classes of mouse homocytotropic antibodies were first described by Nussenzweig, Merryman, and Benacerraf (1964) and have been characterized as an IgG_1 and IgE (Ovary and Warner, 1972; Prouvost-Danon, Binaghi, Rochas, and Boussac-Aron, 1972; Schwartz and Levine, 1973). Mouse IgE is similar to human IgE in its electrophoretic mobility, molecular weight, tissue fixing capacity, and concentration in serum and therefore has been studied more extensively than IgG_1.

IgE production is favored by low doses of antigen adsorbed to

aluminum hydroxide gel (Levine and Vaz, 1970), and several groups have described differences in reagin production in different mouse strains suggesting a genetic component to the response (Revoltella and Ovary, 1969; Levine, 1971). Dorf, Newburger, Hamaoka, Katz, and Benacerraf (1974) have mapped the responder gene to ragweed in the region of H-2K and Ir-1. There is also a genetic component to allergy in humans although there is no direct evidence to link inheritance to HL-A genes (Marsh, Bias, and Ishizaka, 1974).

T and B Cell Collaboration - General Comments:

Collaboration between T and B lymphocytes is required for production of IgM and IgG antibodies to most antigens (reviewed by Katz and Benacerraf, 1972; Claman and Mosier, 1972; Raff, 1973). Using protein antigens coupled with defined haptens such as dinitrophenyl (DNP), it has been shown that T and B lymphocytes recognize different portions of the antigen; T cells recognize a portion of the carrier molecule and B cells recognize the hapten which is also the determinant which combines with serum antibody. To obtain an optimum secondary response to a hapten-carrier conjugate, both hapten and carrier primed cells are required (Rajewsky, 1971; Mitchison, 1972). Furthermore, hapten and carrier determinants must be physically linked under most conditions, suggesting that antigen plays a role in enhancement of necessary cell interactions.

Carrier and hapten recognition events occur at the surfaces of lymphocytes since insolubilized antigens will induce immune responses

in vitro (Feldmann, Greaves, Parker, and Rittenberg, 1974). The specificity of recognition is determined by surface receptors (discussed by Playfair, 1974). Receptors on B cells are immunoglobulins and apparently have the same idiotype and class as the secreted antibody; the average affinity of B cell receptors and serum antibody increases after immunization and is higher with low than with high immunizing doses (Bullock and Rittenberg, 1970; Andersson, 1972; Claflin, Merchant, and Inman, 1973). There have also been several reports that T cell receptors show avidity maturation (Jokipii and Jokipii, 1974; Gorczynski and Rittenberg, 1975). However, the nature of the T cell receptor has still not been firmly established. There is some evidence to suggest it may be immunoglobulin, at least the variable portion, which remains partially buried in the cell membrane and is not susceptible to interaction with anti-immunoglobulin antibodies until after activation by antigen. There are also several investigators who would like to suggest that the T cell receptor is the product of the immune response (Ir) gene (discussed by Katz and Benacerraf, 1975).

Several models have been proposed to explain collaboration between carrier-reactive T cells and hapten-specific B cells. Taussig and coworkers have isolated an antigen-specific factor which is released from activated T cells and allows B lymphocytes to respond specifically in an adoptive transfer system (Taussig, 1974a; Munro, Taussig, Campbell, Williams, and Lawson, 1974). This cooperative factor is absorbed out with antibody directed against the K region of the histocompatibiligy gene locus (H-2) and could thus be a product of an Ir

gene. Another explanation has been provided by the experiments of Feldmann and coworkers (reviewed by Feldmann, Basten, Boylston, Erb, Gorczynski, Greaves, Hogg, Kilburn, Kontianinen, Parker, Pepys, and Schrader, 1974) which demonstrate the release of an antigen-antibody complex (Ag-IgT) from T cells, the subsequent binding of this complex by macrophages, and an antibody response of B lymphocytes to the macrophage-presented antigen. Antibody production does not occur if B cells are incubated with Ag-IgT alone, with macrophages plus antigen alone, or with T cells plus antigen alone; macrophage presentation of antigen complexed with a soluble T cell immunoglobulin is essential. This suggests that besides interaction with specific antigen, B cells require a second signal in order to differentiate into plasma cells.

The nature of the second signal has been the goal of considerable research. Thymus-dependent antigens can induce B cell responses only in the presence of T cells and macrophages. However, the thymus-dependent signal has been replaced in vitro by several soluble products. First, in the absence of T cells, B cell mitogens induce polyclonal antibody synthesis (Moller, 1975); however, recent evidence suggests that mitogen help may be restricted to particulate antigens (Waldmann and Munro, 1975). Second, supernatants from T cells activated by mitogens (Dutton, 1975) antigen (Munro, Taussig, Campbell, Williams, and Lawson, 1974), or allogeneic cells (Schimpl and Wecker, 1975) allow B cell responses to antigen. Third, there are some data to support the role of the third component of complement as an induction signal (Hartmann, 1975). Finally, agents which increase cyclic GMP activate B cells whereas increases in cyclic AMP can induce tolerance

(Watson, 1975).

In contrast to thymus-dependent antigens, thymus-independent antigens appear to provide both the signal achieved by interaction of antigen with the B cell receptor and also the second differentiation signal; this may be a result of their highly repetitive structures (Feldmann and Basten, 1971; Klaus, Janossy, and Humphrey, 1975). Thymus-dependent antigens can become thymus-independent if they are coupled to insoluble matrices in a proper concentration (Feldmann, Greaves, Parker, and Rittenberg, 1974).

When conditions are such that the second signal is not provided in the proper quantity or time sequence, tolerance can occur (Louis, Chiller, and Weigle, 1973; Feldmann, et al., 1974; Bretscher, 1975). It has been shown that under physiological conditions T cell tolerance is more easily induced than B cell tolerance (reviewed by Weigle, 1973). Elson and Taylor (1975) suggested that T cell tolerance is sufficient for maintenance of unresponsiveness to self antigens and that B cell tolerance resulting from direct interaction with antigen most likely occurs only rarely when there is a massive antigen load.

T and B Cell Cooperation in the IgE Response:

In mice it has been demonstrated that the IgE response also requires cooperation between T and B lymphocytes. Michael and Bernstein (1973) were unable to induce a primary IgE response to several protein antigens in nu/nu athymic mice unless the mice had been reconstituted with BALB/c thymocytes. Okudaira and Ishizaka (1973) demonstrated

that mice primed with DNP-Ascaris (DNP-Asc) responded significantly to a secondary challenge with DNP-Ovalbumin (DNP-OV) only if they had also been primed with the heterologous carrier (OV). Furthermore, Kishimoto and Ishizaka (1975) showed that T cell supernatants, harvested from immune lymphocytes challenged in vitro with carrier, could substitute for carrier-primed cells. Taniguchi and Tada (1974) and Okumura and Tada (1974) have also isolated a helper supernatant fraction which is a carrier specific immunoglobulin with a molecular weight of about 160,000. This is similar to the helper IgT activity described by Feldmann, et al. (1974) but distinct from that described by Munro, et al. (1974) which seemed to be an Ir gene product.

Hamaoka, Katz, and Benacerraf (1973) and Hamaoka, Katz, Bloch, and Benacerraf (1973) also demonstrated the need for both hapten and carrier primed cells using an adoptive transfer system; treatment of carrier primed cells with anti-theta and complement before transfer abrogated the secondary response to the hapten-carrier complex.

Many factors influence both IgG and IgE responses in positive and negative ways. However, the IgE response appears to be more sensitive to regulatory influences, and this has raised the question of whether there are distinct subpopulations of both B and T cells, those involved in IgE production and those involved in IgG production.

Kishimoto and Ishizaka (1972b) tried to inhibit an in vitro secondary IgE response of rabbit lymph node cells to DNP-Asc by treatment with antiserum against immunoglobulin heavy chains to eliminate populations of plasma cells. They demonstrated that antibody to the gamma chain could inhibit the IgG response but had no effect on the IgE response; anti-Fab inhibited all antibody production.

They concluded that different B cells are producing IgE and IgG antibodies.

Immunofluorescent staining studies have also suggested that IgE-producing cells synthesize only one class of immunoglobulin. Tada and Ishizaka (1970) and Brown, Borthistle, and Chen (1975) found IgE and IgA on distinct populations of primate lymphocytes. However, double labeling experiments with antibodies against mu or gamma chains and IgE have not been performed.

Whether or not there are two groups of T cells has not been resolved. Dissociation of IgE and IgG responses has led Ishizaka's group to believe there are T cells which collaborate only with IgG-B cells and T cells which collaborate only with IgE-B cells; these T cell populations might have different receptor specificities and/or functional differences. In Ishizaka's experiments with rabbit lymph node cells, a secondary IgE response to hapten-heterologous carrier could be induced only if cells had been primed with the heterologous carrier on alum; carrier priming in complete Freund's adjuvant (CFA) allowed only an IgG secondary response (Kishimoto and Ishizaka, 1973a). Kishimoto and Ishizaka (1973b) have also separated their T cell enhancing supernatant into two fractions; one fraction of 20,000 to 40,000 molecular weight allows DNP-primed cells to produce IgG upon challenge with DNP on a heterologous carrier, and the other fraction of 160,000 molecular weight enhances the IgE response. These two factors are not always produced together suggesting two T cell populations may be involved.

The observation of Jarrett and Ferguson (1974) that Nippostrongylus

brasiliensis enhances the IgE but not the IgG response, and only in rats with intact thymuses, may support Ishizaka's hypothesis of two populations of T cells; only IgE-T cells may be susceptible to augmentation by this nematode. On the other hand, their results could also be interpreted as greater sensitivity of IgE-B cells to nonspecific T cell factors.

This alternative explanation is favored by Hamaoka, Katz, and Benacerraf (1973) and Hamaoka, Newburger, Katz, and Benacerraf (1974) who have shown that adjuvant dissociation of the IgE and IgG responses occurs only with some antigens; dissociation could reflect a lower threshhold for activation of IgE-B cells. This possibility was supported by their observations that primary IgE but not IgG responses could be induced in vitro and that challenge with hapten and carrier on separate molecules was effective for stimulating a secondary IgE response. Newburger, Hamaoka, and Katz (1974) also reported that IgE responses could be enhanced with 100 fold lower doses of lipopolysaccharide (LPS) than were necessary for enhancement of the IgG response. Since LPS augmentation was dependent on T cells, they interpreted this to mean that IgE-B cells are more susceptible to T cell help. The results of Okudaira and Ishizaka (1975) further support a lower threshhold of IgE-B cells; anti-theta treated spleen cells, cultured with hapten-heterologous carrier for 24 hours at 37° before adoptive transfer, could mount an IgE anti-hapten response. They did not feel that differences between IgE and IgG-B cell receptor affinities could account for their results since they had previously shown that optimum antigen doses for in vitro secondary responses

were equivalent for IgE and IgG (Kishimoto and Ishizaka, 1972a).

The results cited above do not answer the question of whether or not there are two populations of helper cells. It seems most likely that a single population demonstrates functional subpopulations due to different requirements of IgE and IgG-B cells for antigen triggering. However, the existence of T cells with different specificities has not been conclusively ruled out.

Suppression of the IgE Response:

The IgE response appears to be more easily suppressed than the IgG response although similar types of suppression have been described for both antibody systems. Passive IgG antibody can inhibit induction of an IgE response (Ishizaka and Okudaira, 1972a; Tada and Okumura, 1971) or an IgG response (Feldmann and Diener, 1972; Hoffman, Kappler, Hirst, and Oettgen, 1974). Under certain conditions, priming with immunogenic doses of carrier can also suppress IgE and IgG responses to hapten on that carrier (Ishizaka and Okudaira, 1973).

Antigenic competition can also influence antibody responses (as reviewed by Pross and Eidinger, 1974). Intramolecular competition as described by Amkraut, Garvey, and Campbell (1966) and discussed by Taussig, Mozes, Shearer, and Sela (1973) may be particularly important in the timothy pollen system where IgE and IgG are directed against different determinants. Taussig, et al. concluded that intramolecular competition is the result of more than one population of B cells competing for a limited number of helper cells.

Direct tolerization of B cells has been described for IgE and IgG systems. Katz, Hamaoka, and Benacerraf (1973) demonstrated long lasting IgE and IgG tolerance induced by DNP coupled to D-GL, a non-metabolizable amino acid copolymer. Tolerance was stable after cell transfer and resembled the receptor blockade described by Aldo-Benson and Borel (1974). Lee and Sehon (1975a, 1975b) also obtained what appeared to be a similar type of B cell tolerance by treatment of mice with DNP-conjugated mouse immunoglobulin (DNP-MGG). They could suppress primary IgE and IgG responses, but only ongoing or secondary responses of the IgE class; this suggested greater sensitivity of IgE-B cells to tolerance induction. Tolerance was stable upon cell transfer, although it was not infectious and therefore did not involve suppressor cells, and the number of IgE antibody forming cells, as measured by heterologous adoptive cutaneous anaphylaxis (Kind and Macedo-Sobrinho, 1973), was actually decreased.

Tolerance at the T cell level has also been described for both IgG and IgE systems, and T cell tolerance can result in B cell unresponsiveness. Ishizaka's group has used antigens modified by treatment with 8M urea to induce specific tolerance or suppression of IgG and IgE responses, apparently at the level of the T lymphocyte (Ishizaka, Kishimoto, Delespesse, and King, 1974; Ishizaka, Okudaira, and King, 1975; Takatsu and Ishizaka, 1975). They showed that urea denatured ragweed pollen or ovalbumin retained carrier but not major hapten determinants. When given intravenously to mice, the altered antigens suppressed primary IgE and IgG responses, and ongoing or secondary IgE responses, to the native antigen. Furthermore, treatment resulted

in decreased numbers of carrier and hapten reactive spleen cells as determined by adoptive transfer experiments; T cell suppression most likely prevented B cell differentiation.

Suppressor T cells and T cell factors have been described which affect IgG production (reviewed by Gershon, 1974) or IgE production. Tada's group showed that the IgE response of rats to DNP-Asc could be prolonged by treatments which depleted T cells; these included irradiation (Tada, Taniguchi, and Okumura, 1971), thymectomy and splenectomy (Okumura and Tada, 1971a), and therapy with anti-thymocyte serum (ATS, Okumura, Tada, and Ochiai, 1974). They concluded that suppressor T cells affecting IgE production were preferentially depleted; on the other hand, these same treatments abrogated helper T cells necessary for IgG and IgM production. Elimination of suppressor T cells was substantiated by the demonstration that an ongoing IgE response could be rapidly suppressed by carrier primed thymocytes, spleen cells, or supernatants from these cells (Okumura and Tada, 1971b; Tada, Okumura, and Tanaguchi, 1973). The suppressive factor could be absorbed out with ATS but not with anti-immunoglobulin (anti-Ig) which distinguished it from the soluble helper factor.

Tada's work suggests that suppression of IgE and IgG responses can be partially dissociated, due to different populations of suppressor T cells, different proportions of helper and suppressor cells, or different sensitivities of B cells.

Another example of dissociation of IgE and IgG responses was obtained by Gollapudi and Kind (1975a, 1975b) who achieved enhanced IgE production by in vivo therapy with doses of Concanavalin A (Con A)

which suppressed the IgM response.

To summarize the above, IgE production is dependent upon cooperation between B and T cells, the latter providing helper and suppressor effects. The cell interactions necessary appear to be similar to those required for IgG production, and tolerance can occur at the level of the T cell or the B cell; B cell tolerance can be induced either directly or as a result of T cell influences. The major difference between the IgG and IgE systems seems to be one of sensitivity; IgE responses are more easily enhanced and suppressed than IgG responses. Although different populations and/or different proportions of T cells (helper and suppressor) may be important, it is much easier to visualize regulation at the level of the B lymphocyte.

In humans allergic to timothy pollen, AgD therapy appears to selectively suppress specific reagin formation, and this is consistent either with the hypothesis that IgE-B cells are more sensitive to tolerance induction or that different populations of T cells are involved. The observation in mice that IgE and IgG responses cannot usually be completely dissociated makes it difficult to distinguish between these possibilities. However, by using different timothy pollen immunogens and different immunization regimes, I have been able to obtain mice which synthesize only IgE as well as mice which synthesize IgE and IgG in different proportions. Hopefully, comparative studies will allow conclusions to be drawn about both the site of AgD suppression and the number of lymphocyte subpopulations responding to timothy pollen antigens.

Lymphocyte Transformation - B and T Cell Responses in Vitro:

In order to examine hapten and carrier specific recognition events and the effects of AgD on them, I elected to use a lymphocyte transformation system which measures the proliferation of primed lymphocytes to antigen stimulation in vitro (Dutton and Eady, 1964). Lymphocyte transformation has been used successfully with several allergens including timothy pollen (Girard, Rose, Kunz, Kobayashi, and Arbesman, 1967; Richter and Naspitz, 1968; Malley, Wilson, Barnett, and Perlman, 1971; Romagnani, Biliotti, Passakeva, and Ricci, 1973; Spengler, de Weck, and Geczy, 1974).

In order to provide information about hapten and carrier specific cells, lymphocyte transformation must measure responses of B and T lymphocytes. When first described, lymphocyte transformation was thought to be an in vitro correlate of cell mediated immunity measuring only T lymphocyte proliferation (reviewed by Valentine, 1971). In systems where there is only a delayed response and no antibody, it may be a good correlate of cell mediated immunity. However, several authors have recently reported B cell proliferation in culture; whether or not B cell division is specific has not been conclusively established.

Several techniques have demonstrated dividing B cells in mixed cultures of T and B lymphocytes. Elfenbein and Rosenberg (1973) and Elfenbein, Shevach, and Green (1973) combined autoradiography and visualization of complement receptor lymphocytes (B cells) to show that B cell blasts occurred after stimulation of immune rabbit

or guinea pig lymph node cells with vaccinia virus or ovalbumin respectively. Kirchner, Oppenheim, and Blaese (1973) and Kirchner, Altman, Fridberg, and Oppenheim (1974) found that lymphocytes from agammaglobulinemic chickens could proliferate in vitro to the sensitizing antigen, keyhole limpet hemocyanin (KLH) or Brucella abortus, but would not elicit chemotactic factors; thus, lymphocytes from chickens without demonstrable T cell function, presumably B cells, could respond in vitro. After immunization with DNP-carrier conjugates, Warnatz, Scheiffarth, and Gollnick (1972) could stimulate mouse spleen cells with free carrier or hapten on the homologous or heterologous carrier; this implied proliferation of distinct hapten and carrier specific cell populations.

In several experiments, the B cell response has been shown to be nonspecific since it occurs to T cell mitogens or unrelated antigens. Vischer (1972) showed that blasts induced by phytohemagglutinin (PHA), pokeweed mitogen (PWM), or KLH stained with fluoresceinated anti-theta or anti-Ig. Geha, Schneeberger, Rosen, and Merler (1973) and Geha and Merler (1974) isolated a soluble factor from human T lymphocytes purified by BSA gradient centrifugation of tonsil cells; the factor was elicited only in the presence of specific antigen, was necessary for the response of B cells, but allowed B cells to respond to a different antigen. Two groups (Gery, Kruger, and Spiesel, 1972; Piquet and Vassalli, 1972, 1973) confirmed the above by using thymectomized, bone marrow-reconstituted CBA mice which had received thymus grafts with the T6T6 chromosomal marker; sheep red blood cells (SRBC) and PHA induced blasts which did not have the T6 marker (B cells).

Furthermore, using a colchicine arrest technique, Piquet and Vassalli (1973) showed that B cell proliferation was dependent on and occurred later than T cell proliferation.

Using mouse spleen cells, separation of immune B and T lymphocytes in vitro and culture of isolated populations has been used to demonstrate B cell division in the absence of T cells. Cell separation has been achieved by selective depletion with anti-theta or anti-Ig and complement (Osborne and Katz, 1973a, 1973b; Snippe, Nab, and van Eyk, 1974; Snippe and van Eyk, 1974) or by anti-Ig columns (Mugraby, Gery, and Sulitzeanu, 1974). Isolated T or B cells from mice immunized to SRBC or DNP-carrier conjugates had only partially reduced in vitro responses to antigen when compared to unfractionated cells. This suggested that both cell types could respond directly and specifically to antigen stimulation. Furthermore, in the experiments of Mugraby, et al., recombined B and T cells showed a synergistic response implicating nonspecific influences as well.

In contrast to the above experiments, two groups have concluded that B cells do not proliferate in vitro. Chess, MacDermott, and Schlossman (1974) separated human tonsil cells on anti-Ig columns, and only T cells responded to mumps, SK-SD, and rubella antigens. In this experiment, the antigens tested all induce primarily cell mediated immunity, and there may not have been any memory B cells; alternatively, these results could support the idea that T cells are required for a B cell response. Rubin and Wigzell (1974) found an increased response of immune guinea pig lymph node cells to antigen following passage over anti-Ig columns to remove B cells. These

results confirm the ability of T cells to respond but do not rule out the ability of B cells to respond. Neither of these studies conclusively refutes those cited above; results may be very dependent on the degree of cell purification achieved, the source of the lymphocytes, and the species studied.

The data thus seem to indicate that B cells can proliferate in vitro. However, whether proliferation is due to direct interaction with antigen, or whether it requires complex interactions with T cells, macrophages, and/or soluble enhancing factors is still not clear. Furthermore, the specificity of the B cell response needs to be firmly established. The regulatory controls discussed earlier which have been observed in Mishell-Dutton and in vivo systems may or may not influence the in vitro lymphocyte transformation system.

Lymphocyte Transformation - Culture Variables:

Several variables do appear to be important to in vitro lymphocyte stimulation, and I would like to briefly mention them to help put the data in perspective.

The requirement for adherent cells or macrophages is controversial. Several investigators have shown that macrophages, or macrophage supernatants, are essential for the response to allogeneic cells (Bach, Alter, Solliday, Zoschke, and Janis, 1970) or PPD (Havemann and Schmidt, 1974), and others have concluded that macrophages are necessary for antigen presentation (Shortman and Palmer, 1971; Waldron, Horm, and Rosenthal, 1973; Rosenstreich and Rosenthal, 1973; Lohrman,

Novikovs, and Graw, 1974). Rosenthal, et al. established that as few as 0.5% macrophages was sufficient for the response of guinea pig peritoneal lymphocytes to antigen. Macrophages or macrophage supernatants are also required for in vitro antibody responses (Mosier and Coppleson, 1968; Aaskov and Halliday, 1971; Hoffman and Dutton, 1971). In contrast, there seems to be little or no requirement for macrophages in the lymphocyte responses to mitogens (Lake, Bice, Schwartz, and Salvaggio, 1971; Hanna and Leskowitz, 1973).

Macrophages have also been shown to have suppressive effects. Yoshinaga, Yoshinaga, and Waksman (1972) demonstrated a complete inhibition of the response of rat lymph node cells to LPS with 2.5 to 5% adherent cells. Calderon, Williams, and Unanue (1974) could inhibit responses to T and B cell mitogens with a supernatant from mouse adherent peritoneal cells. Opitz, Niethammer, Lemke, Flad, and Huget (1975) also isolated an inhibitory macrophage supernatant, the generation of which required contact with lymphocytes; they postulated that the inhibitor was free thymidine which was merely competing with labeled thymidine for incorporation into DNA. Therefore, the ratio of macrophages to lymphocytes and/or the source of macrophages may be critical.

Besides macrophages and macrophage factors, lymphocyte products may also contribute to the overall proliferative response; these could be specific or nonspecific. Several enhancing T cell factors have already been mentioned (and see review, Dumonde, Page, Matthew, and Wolstercroft, 1972). The enhancing effect of LPS on responses to Con A and PHA (Schmidtke and Najarian, 1975; Ozato, Adler, and Ebert,

1975) may also suggest the existence of enhancing B cell factors.

The last soluble product deserving mention is antibody which could be produced during culture. Excess soluble antibody can inhibit lymphocyte transformation when added at the initiation of culture, and antigen-antibody complexes can enhance the proliferative response (Oppenheim, 1972; Thorbecke and Siskind, 1973; Romagnani, Biliotti, and Ricci, 1975).

The final question relating to the lymphocyte transformation system is whether it has any meaning in terms of the immune response of the intact animal. The magniture of lymphocyte transformation in a single lymphoid organ does not seem to correlate with serum antibody titers although it may correlate with delayed skin tests (Valentine, 1971). Lymphocytes distal to the immunizing site respond later than those in draining lymph nodes (Vischer and Stastny, 1967). If all lymphoid tissue were assayed, the magnitude of response might correlate with serum antibody, or with immunologic memory.

Whether affinity differences or alterations of lymphocyte receptors are reflected by shifts in dose response curves or hapten inhibition profiles is controversial. Bast, Manseau, and Dvorak (1971) showed that with increasing time after immunization, decreasing doses of human serum albumin (HSA) antigen induced optimum stimulation of guinea pig lymph node cells and maximum responses increased; they concluded that affinity maturation of lymphocyte receptors was occurring. Also using HSA-immune guinea pig lymph node cells, Rubin and Wigzell (1974) could not show changes in dose response curves but demonstrated shifts in hapten inhibition profiles; although these changes could

reflect affinity maturation, they felt they were more likely due to increased numbers of antigen reactive cells. Cohen and Paul (1974) found that any decreases in the optimum antigen dose needed for stimulation of immune guinea pig peritoneal cells were due to increased avidities of macrophage cytophilic antibodies and not due to increased avidities of lymphocyte receptors; they did attribute increases in the magniture of response to lymphocyte clonal proliferation in the host. Finally, Walters, Moorhead, and Claman (1974) could not show any differences in the optimum stimulatory antigen dose, or the amount of hapten required for inhibition, with mouse spleen cells obtained from animals that had been immunized with different doses of hapten conjugated to isologous proteins; however, avidity differences were demonstrable at the level of the plaque forming cell. Possibly the lack of carrier reactive T cells contributed to these observations, or the plaque forming system could be more sensitive than lymphocyte transformation.

With an understanding of the variables affecting the lymphocyte transformation system, and the problems of relating it to the in vivo situation, it can be a useful tool. It proved valuable for approaching some of the questions posed in this Introduction. With spleen cells from mice immunized to timothy pollen antigens it was first necessary to determine whether both B and T lymphocytes respond in culture since this question has not been conclusively answered. B and T cell populations, isolated by a procedure which has not been used by other authors referenced, did respond specifically to antigen, and this allowed cell interactions and antigen specific recognition events to

be studied. The IgE system appears to be highly thymic-dependent and more sensitive to regulatory influences than the IgG system; it should therefore provide a valuable model for looking at regulation of immunoglobulin synthesis. By comparing responses of B and T cells from mice immunized to produce predominantly IgE or IgG antibody, I hoped to detect differences in cell populations, e.g. differences in specificities, receptor avidities, or population sizes as measured by the magnitude of responses. Such information might add to the controversy of whether or not there are distinct populations of T cells collaborating with IgE and IgG-B cells. Finally, cellular recognition events could be evaluated in vitro to determine the number of cell populations responding to distinct pollen determinants and whether AgD has a carrier determinant. As a model for hapten suppression this would provide a means of assessing the site(s) of AgD suppression in vitro and indicate its mechanism of action in vivo as well.

MATERIALS AND METHODS

Animals:

Femals LAF_1 and C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) of 8 to 12 weeks of age were used for antibody production; LAF_1 mice served as sources of normal and immune spleen cells. Six to eight week old female Swiss Webster mice (Simonsen Laboratories, Gilroy, California) were used for passive cutaneous anaphylaxis (PCA).

New Zealand White rabbits (10 to 15 kg) were obtained from Arthur Fessler, Hillsboro, Oregon.

Antigens:

Crude timothy pollen extract (WST) and its major antigens were purified as described by Malley and Harris (1967). Briefly, ether-defatted pollen was extracted with phosphate buffer and dialyzed against distilled water to yield the nondialyzable WST and a dialysate. A mixture of the two major antigens, Antigen A and B (AgA+B), was purified from WST by ammonium sulfate precipitation and ethanol fractionation. AgA and AgB were separated from one another by salt-gradient elution from DEAE-Cellulose or by immunoelectrofocusing. Both fractions are glycoproteins. The molecular weight of AgA is about 13,000 and that of AgB about 10,500 daltons. The major antigenic determinants of these antigens are distinct although AgA also has a small amount of the AgB determinant.

The low molecular weight fraction, Antigen D (AgD), was isolated from the dialysate by Sephadex G-50 gel filtration. AgD has been characterized and consists of a glycopeptide-flavonoid pigment with an average molecular weight of 5,000 daltons. AgD is unable to induce antibody precipitation, PCA, or histamine release from monkey lung tissue sensitized with human serum from timothy pollen allergic patients; however, AgD will inhibit these reactions. Inhibition is specific for the AgB determinant. Therefore, AgD is monovalent for the AgB hapten (Malley and Harris, 1967).

Antigen D was further separated into three fractions on a 2.5X100 cm Bio-gel P-2 column. These fractions have been designated D-1, D-2, and D-3 by their order of elution, and their molecular weights were estimated to be 5,000, 2,500, and 800 daltons respectively. All three fractions will inhibit PCA or histamine release using the monkey lung tissue assay and therefore possess the AgB determinant[1].

Two preparations of Ascaris suum were used. The crude extracts used for PCA challenge were gifts from W.E. Vannier (Department of Naval Research, Bethesda, Maryland) and G. Strejan (University of Western Ontario, London, Ontario, Canada). A purified fraction of the crude extract was prepared by Sephadex G-200 and DEAE-Cellulose chromatography according to the method of Hussain, Bradbury, and Strejan (1973).

Antigen B was conjugated to the purified fraction of Ascaris by the carbodiimide procedure (Likhite and Sehon, 1967). Purified

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1. A. Malley, unpublished observations

Ascaris (25mg) and AgB (75mg) were mixed with 400mg carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl, Story Chemical corporation, Muskegon, Michigan) for 30 minutes at room temperature in a total volume of 14ml. The AgB-Ascaris (AgB-Asc) conjugate was separated from unreacted materials by Sephadex G-75 gel filtration. The molar ratio of AgB to Ascaris was estimated spectrophotometrically to be 3 to 1 by comparing absorbance at 404nm, which detects the pollen pigment, with absorbance at 280nm. The AgB-Asc conjugate precipitated with rabbit antisera against WST, AgB, and Ascaris, and it was just as effective as the original purified Ascaris for inducing PCA reactions in mice sensitized with mouse anti-Ascaris serum. The Ascaris portion of the conjugate will arbitrarily be referred to as the carrier, and the AgB portion as the hapten.

All antigens, except D-3 which has only a single amino acid, were quantified for protein content by Nesslerization (Campbell, Garvey, Cremer, and Sussdorf, 1970). D-3 was standardized by absorbance at 404nm.

Sheep red blood cells (SRBC) were obtained weekly in sterile Alsevers solution from Sheep Blood Supply, Tualatin, Oregon. For immunization and hemagglutination they were used at 2 to 4 weeks of age after three washes in Dulbecco's phosphate buffered saline (PBS); for preparation of EA monolayers they were used within one week after bleeding.

Mitogens:

Concanavalin A (Con A, Miles Laboratories, Kankakee, Illinois) was made to 100μg/ml in unsupplemented culture medium or distilled water, sterilized, and stored at -20°; it could be thawed up to three times with little loss of activity. Purified phytohemagglutinin (PHA-P, Difco Laboratories, Detroit, Michigan) was diluted with sterile distilled water according to the manufacturer's directions and frozen in 0.1ml aliquots; a 1/100 dilution of the stock is referred to as a 1% solution. Salmonella typhimurium lipopolysaccharide (LPS, Difco No. 594136) was stored as a sterile stock solution at 100μg/ml in distilled water. Neither PHA nor LPS was thawed more than once.

Immunization:

For the production of homocytotropic antibodies, I followed the procedure of Levine and Vaz (1970). Mice were immunized intraperitoneally (ip) with 1 or 10μg of WST, purified Ascaris, or AgB-Asc adsorbed to 1mg of aluminum hydroxide gel (alum). Animals were boosted every third week with the same dose used for priming, and groups of 3 to 5 animals were bled via heart puncture at various times after immunization. Sera were pooled for assay.

Immunogenicity of AgD was evaluated by ip immunization every 3 weeks with 100μg, 500μg, or 1mg of AgD in 0.2ml of alum, complete Freund's adjuvant (CFA), saline, or 2% alginate. Alginate was a gift from Dr. F. Perlman, Portland. Groups of mice were bled one week following the second, third, and fourth immunizations.

For the production of precipitating antibodies, mice were immunized ip with 0.1ml containing 250μg of WST with an equal volume of CFA or $5X10^8$ SRBC in 0.2ml saline. Animals were boosted at 2 to 4 week intervals.

Rabbits were immunized at multiple sites every one to four weeks with either 38mg of AgA+B or 10mg of WST in an equal volume of CFA. Animals were bled out one week following the fourth immunization. These two serum pools (RaAgA+B and RaWST) were heat inactivated at 56^o for 30 minutes and used as reference sera.

For production of rabbit antibody to SRBC (RaSRBC), rabbits were immunized ip with 1ml of a 30% suspension of SRBC and boosted iv every 2 weeks with 1ml of a 50% SRBC suspension. Serum obtained one week after the fourth immunization was heat inactivated and had a hemagglutination titer of 5120.

Rabbit anti-brain associated theta serum (RaBAθ) was produced according to the method of Gyongyossy and Playfair (1973). The equivalent of three C3H brains emulsified in CFA was injected subcutaneously into three sites; each animal was boosted after 2 weeks with 3 C3H brains in incomplete Freund's adjuvant (IFA) and bled out 10 days later. Serum was heat inactivated and adsorbed twice with mouse liver powder (100mg/ml serum; Colorado Serum, Denver, Colorado).

Sheep antibody to mouse theta (Saθ) was a gift from Marilyn Baltz. It was raised by immunization with 5 BALB/c brains in CFA followed by an iv boost at 26 days with $2.2x10^8$ thymocytes. Sheep were bled 9 and 23 days after boosting, and equal volumes of serum (No. 144, 11-6 and 11-20-74) were pooled, heat inactivated, and

adsorbed by passage over a normal mouse serum (NMS) immunoadsorbent.

Tissue Culture Medium:

Powdered RPMI-1640 with bicarbonate buffer and 25mM L-glutamine (Grand Island Biological Company, Berkeley, California) was reconstituted, brought to pH 7.2, sterilized by membrane filtration, and supplemented with 5% heat inactivated fetal calf serum (FCS, GIBCO) and antibiotics (50U/ml penicillin and 50μg/ml streptomycin, GIBCO). In some experiments, medium was further supplemented with HEPES (Calbiochem, San Diego, California) or 2-mercaptoethanol (2-ME, Matheson, Coleman, and Bell, Norwood, Ohio).

Assays for Antibody Activity:

Both IgG_1 and IgE homocytotropic antibodies were assayed by passive cutaneous anaphylaxis (PCA), the former by antigen challenge 3 hours after serum implantation and the latter by challenge at 48 hours (Clausen, Munoz, and Bergman, 1969). Serial dilutions of antiserum, beginning with a 1/10 or 1/50, were prepared, and three serum dilutions in volumes of 0.025ml were injected intradermally (id) into the dorsal sides of each Nembutal anesthetized mouse; each dilution series was repeated in three mice. Animals were challenged iv at 3 or 48 hours with WST (100μg), crude Ascaris extract (330μg), AgB (50μg), or AgA (50μg) in 0.2ml of a 0.5% solution of Evan's blue dye (Allied Chemical Company, New York, New York). Thirty minutes

after challenge the animals were killed and the bluing reactions measured on the everted skin surfaces. Antibody titers are expressed as the reciprocal of the highest dilution giving a 5X5mm reaction in 3 test animals. If an endpoint was not reached, other dilutions were tested.

Antigen D inhibition of PCA was evaluated by preincubating serum dilutions with increasing amounts of AgD before PCA assay; preincubation was for 2 hours at 37° followed by 16 hours at 4°. The concentration of AgD was adjusted to provide the equivalent of 10 to 200μg per implantation site of 0.025ml. Test animals were challenged at 3 or 48 hours with 25μg of WST. Control animals were challenged with 330μg of crude Ascaris extract to evaluate the specificity of inhibition or at 0, 1, 3, or 48 hours with dye only to evaluate leakage resulting from AgD itself.

Immunodiffusion was performed on microscope slides covered with 3ml of 0.85% Noble agar (Difco) in saline. Diffusion wells (3mm in diameter) were separated by 3mm. Test serum was used straight or after dilution in PBS. Slides were incubated at room temperature in a humidified desiccator for 6 to 48 hours.

Hemagglutination titers were determined in round bottomed microtiter plates (Cooke Engineering Company, Los Angeles, California) with 0.5% bovine serum albumin (BSA, Sigma Chemical Company, St. Louis, Missouri) in PBS as diluent. Equal volumes of test serum dilutions and 0.25% SRBC were incubated for sixty minutes at 37°. The hemagglutination titer is the reciprocal of the highest

serum dilution giving visible agglutination.

Anti-theta sera were assayed for cytotoxicity according to the procedure of Boyse, Old, and Chouroulinkov (1964). Spleen cells, bone marrow cells, or thymocytes from C3H or LAF_1 mice were suspended at 10^7 cells/ml in RPMI supplemented with 5% FCS. Cells (0.1ml) were incubated with 0.1ml of a dilution of anti-theta serum or diluent control for 45 minutes at 4 or 37°, washed once, and incubated for an additional 45 minutes at 37° with 0.2ml of a 1/3 dilution of guinea pig complement adsorbed with agarose (Cohen and Schlesinger, 1970). For viability determinations, 0.025ml of a 1% solution of trypan blue (Allied Chemical Company) was added to each sample and total cells and % viable were counted in a hemacytometer. Results were calculated in two ways:

$$\text{cytotoxic index (CI)} = \frac{\%\text{ dead E} - \%\text{ dead C}}{100 - \%\text{ dead C}} \times 100, \text{ or}$$

$$\%\text{ remaining viable} = \frac{\%\text{ viable E} \times \text{total remaining cells E}}{\%\text{ viable C} \times \text{total remaining cells C}} \times 100$$

where E = cells + antibody + complement, and

C = cells + complement only.

Separation of B and T Lymphocytes:

B and T lymphocytes were separated by the method of Kedar, Ortiz de Landazuri, and Bonavida (1974) with slight modifications. Falcon petri dishes (number 3002, 60X15mm) were treated for 45 minutes at room temperature with 2.5ml of poly-L-lysine (PLL, Type I-B, approximate molecular weight 70,000; Sigma Chemical Company) at 100μg/ml

in sterile 0.15M PBS, pH 7.2. Plates were washed extensively with PBS and then incubated for 45 minutes at room temperature with 2.5ml of a 1.5% solution of SRBC. At this point, monolayers could be stored for up to 4 days at 4^{o}. For final preparation of the EA (SRBC-aSRBC) complexes, washed monolayers were incubated for 60 minutes at 37^{o} with a 1/150 dilution of RaSRBC (hemagglutination titer = 5120); plates were washed with PBS and RPMI before use.

Normal or immune LAF_1 spleen cell pools were prepared by flushing culture medium through spleens with a 1cc syringe and a 25 gauge needle. Single cell suspensions obtained by successive passage through a 25 gauge needle were washed three times in RPMI. Total cell counts were performed in a hemacytometer with crystal violet stain; viability was determined by trypan blue exclusion and was always greater than 98%. Cells were adjusted to 10^{7} mononuclear cells/ml in supplemented RPMI.

For adsorption, 2.5ml of a spleen cell suspension was added to each monolayer and incubated for 60 minutes at 37^{o}; for the first 30 minutes dishes were rocked at 5 cycles per minute, and for the last 30 minutes they were kept stationary in 5% CO_2, 95% air to help maintain neutral pH.

The unbound cells were removed with a Pasteur pipette and pooled with the first wash of the monolayers; this fraction will be referred to as T cells. The bound fraction, referred to as B cells, was recovered after one additional wash and lysis of the monolayers with 3ml of Tris-buffered ammonium chloride (Boyle, 1968). Fractionated cells were washed once with RPMI, counted, and adjusted to the proper

cell concentrations for lymphocyte transformation or cytotoxicity assay. Viability of T cells was usually greater than 98% and viability of B cells averaged 95%.

Lymphocyte Transformation:

The lymphocyte transformation assay was performed according to the micro method of Strong, Ahmed, Thurman, and Sell (1973) with spleen cells or purified lymphocytes adjusted to $6X10^6$ mononuclear cells/ml unless otherwise indicated.

With a Hamilton repeating syringe, 0.1ml of spleen cells or purified cells and 0.1ml of the appropriate mitogen, antigen, or medium control were dispensed into flat bottomed wells of Falcon 3040 Microtest II tissue culture plates. Loosely covered plates were incubated at 37° in a 5% CO_2, humidified atmosphere. Twenty-four hours before harvest, each culture well was pulsed with 1.0μCi thymidine-methyl-^3H (6.7Ci/mmole, New England Nuclear, El Cerrito, California). At the times indicated in the Results section, cultures were harvested with automated sample harvester (Hollyhill Laboratories, Hillsboro, Oregon). Cells were deposited on glass fiber filter paper (Reeve Angel, Clifton, New Jersey) and washed extensively with 0.85% saline; measurement of thymidine uptake correlates with thymidine incorporation into TCA-precipitible DNA[2]. After drying, filter papers were added to 3ml of toluene-Permafluor (Packard Instrument Company, Downers Grove, Illinois) and counted in a Packard 3320 liquid scintil-

2. G. Thurman, personal communication

lation counter. Each variable was evaluated in triplicate, and the results calculated by an XDS 920 computer as average cpm/culture ± the standard error (S.E.). In some experiments stimulation indices are reported:

stimulation index (S.I.) = cpm E/cpm C

where E = cultures containing mitogen or antigen, and

C = cultures with medium only (cell background).

When AgD was added to cultures, it was dispensed in a volume of 0.05ml, and antigen or medium control was also added in 0.05ml. Inhibition was calculated as:

$$\frac{\%\text{ inhibition E} - \%\text{ inhibition C}}{100 - \%\text{ inhibition C}} \times 100$$

$$\text{where } \%\text{ inhibition E} = \frac{\text{cpm cells + AgD + antigen}}{\text{cpm cells + antigen only}} \times 100$$

$$\text{and } \%\text{ inhibition C} = \frac{\text{cpm cells + AgD only}}{\text{cpm cells + medium only}} \times 100.$$

For some experiments, cells were irradiated prior to culture with a Thermax Therapy Tube operating at 125kv and 5ma and delivering an air dose of 241R/minute (3000R total dose).

RESULTS

Antibody Production:

Optimum conditions for production of IgG_1 and IgE homocytotropic antibodies were defined in LAF_1 and C3H mice. I wanted to compare responses to WST and to the major allergen in humans, AgB. However, AgB is a poor immunogen in other species[3], and I therefore first determined whether Ascaris might serve as a good carrier for facilitating a homocytotropic antibody response to AgB covalently coupled to it. Ascaris was chosen because it induces high titers of homocytotropic antibodies in all other species tested.

Figure 1 shows the IgG_1 and IgE responses of LAF_1 mice immunized with 1 or 10μg doses of purified Ascaris adsorbed to alum. Both responses were boosterable and were maximum after 4 immunizations. These results suggested that Ascaris would be a good carrier molecule.

Therefore, responses of LAF_1 and C3H mice to AgB-Asc or WST were compared. Mice were immunized every 3 weeks with either 1μg of AgB-Asc or 10μg of WST on alum. Since about 10% of WST is antigenic, there is approximately two and one half times more timothy antigen in 10μg of the crude preparation than in 1μg of the conjugate; however, not all of this antigenic material is AgB.

Figure 2 shows the IgG_1 responses measured at weekly intervals. The IgG_1 response to timothy pollen antigen was similar when LAF_1 mice were immunized with WST or AgB-Asc (Figure 2a); the maximum titers

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3. A. Malley, unpublished observation

Figure 1: IgG_1 and IgE responses to Ascaris. LAF_1 mice were immunized with 1 or 10μg of Ascaris adsorbed to alum at the times indicated by the arrows and bled one week following the second, third, and fourth immunizations. IgG_1 and IgE titers were determined by challenge of recipient mice with 100μg of WST at 3 and 48 hours respectively; titers are the reciprocals of the highest dilutions giving 5X5mm bluing reactions in 3 test animals. IgE titers are shown by the open circles (1μg Asc immunizing dose) or closed circles (10μg Asc immunizing dose) and IgG_1 titers by the open squares (1μg Asc immunizind dose) or closed squares (10μg Asc immunizing dose).

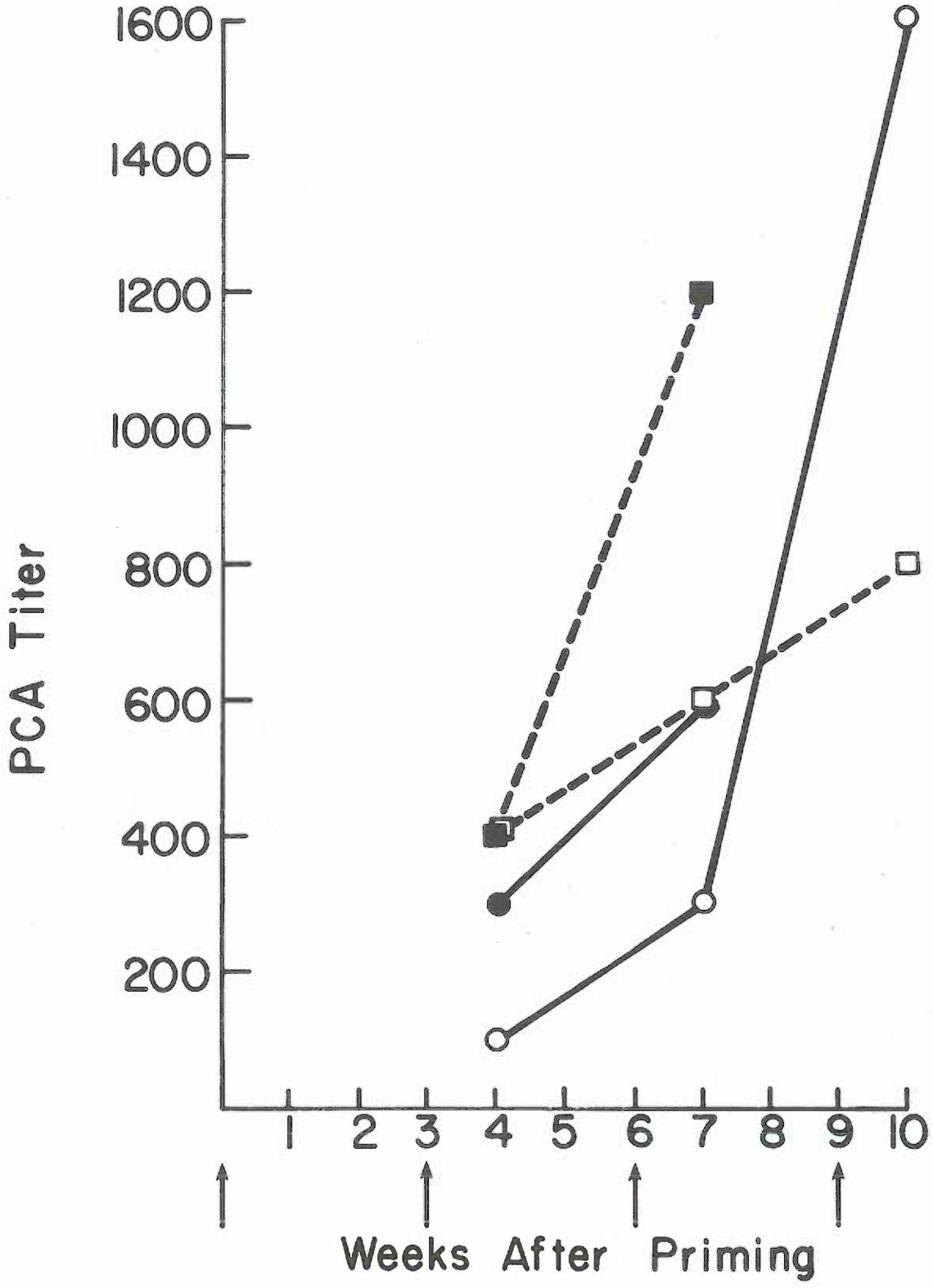
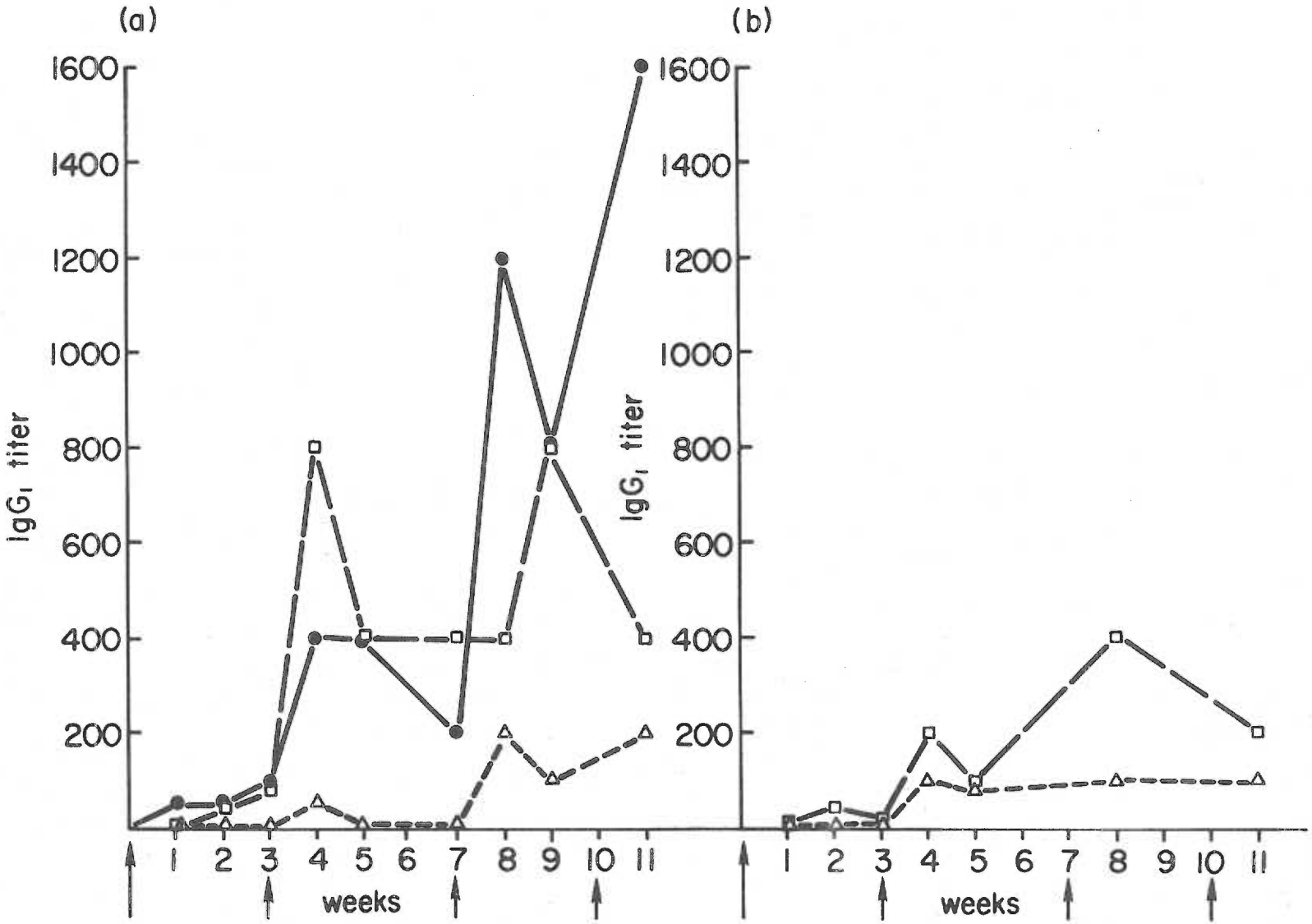

1600
1400
1200
1000
800
600
400
200
PCA Titer
1
2
3
4
5
6
7
8
9
10
Weeks After Priming

Figure 2: Kinetics of the IgG_1 response. LAF_1 (a) or C3H (b) mice were immunized with 10μg of WST on alum or 1μg of AgB-Asc on alum at the times indicated by the arrows. Antibody titers were determined by PCA challenge of recipient mice at 3 hours with 100μg of WST or 330μg of crude Ascaris extract; titers represent the reciprocals of the highest serum dilutions giving 5X5mm bluing reactions in three test animals. Closed circles and open squares illustrate anti-timothy titers of mice immunized with WST or AgB-Asc respectively. Open triangles represent anti-Ascaris titers of mice immunized with AgB-Asc.

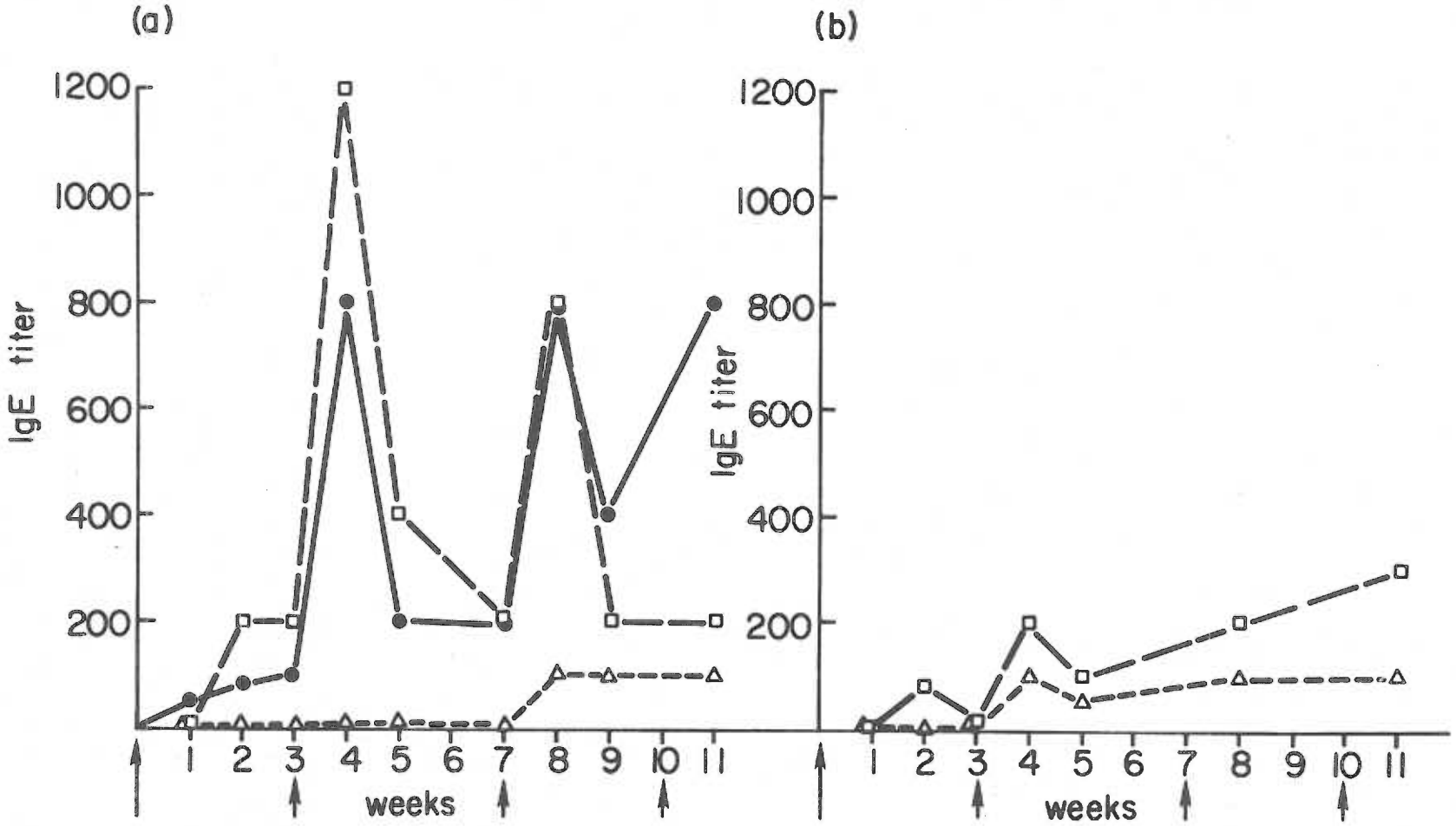

(a)
(b)
IgG1 titer
1600
1400
1200
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11
weeks

were 1600 and occurred one week following the second or third immunization. Although not shown, animals bled one week after a fifth, sixth, or seventh immunization did not show titers greater than 1600 although responses always showed a booster effect. Figure 2a also shows that with AgB-Asc as the immunogen, the response to Ascaris carrier was lower and came up later than the response to the AgB hapten. Furthermore, the maximum anti-carrier response was four times lower than when unconjugated Ascaris was used for immunization (Figure 1).

The IgG_1 responses of C3H mice immunized with 1μg AgB-Asc are shown in Figure 2b; titers were determined weekly for 5 weeks and then one week after each subsequent boost. The general pattern of response was similar to that of LAF_1 mice except that antihapten titers were consistently lower, the maximum being 800 in LAF_1 mice versus 200 in C3H mice.

Figure 3 shows the IgE responses of LAF_1 and C3H mice to AgB-Asc and WST. The IgE response was similar to the IgG_1 response in terms of kinetics and magnitude. Both WST and AgB-Asc induced similar peak IgE titers of 800 and 1200 to the timothy determinant in LAF_1 mice (Figure 3a). With AgB-Asc as the immunogen, the anticarrier response was lower and occurred later than the anti-hapten response. The maximum titer to the Ascaris carrier was 100 whereas when Ascaris alone was used for immunization the titer at 10 weeks was 1600. C3H mice showed a four fold lower antihapten IgE response than LAF_1 mice (Figure 3b). Therefore, LAF_1 mice were used for all subsequent experiments.

Figure 3: Kinetics of the IgE response. LAF_1 (a) or C3H (b) mice were immunized with 10μg of WST on alum or 1μg of AgB-Asc on alum at the times indicated by the arrows. Antibody titers were determined by PCA challenge of recipient mice at 48 hours with 100μg WST or 330μg of crude Ascaris extract; titers represent reciprocals of the highest dilutions giving 5X5mm reactions in three test animals. Closed circles and open squares illustrate anti-timothy titers of mice immunized with WST or AgB-Asc respectively. Open triangles represent anti-Ascaris titers of mice immunized with AgB-Asc.


(a)
(b)
IgE titer
1200
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11
weeks

Precipitating antibodies to WST and Ascaris in these same groups of mice were followed by immunodiffusion. There were no detectable antibodies against WST or Ascaris until one week after a secondary immunization. Precipitating antibody was then demonstrable at one and two weeks, but was negligible at three weeks, following each boost.

Effects of changing antigen dose and adjuvant were investigated cursorily to see if IgE and IgG responses could be dissociated. Table I lists the maximum IgE and IgG_1 titers obtained with different immunization regimes. Animals immunized with 250μg of WST in CFA produced very little homocytotropic antibody, and the response could not be boosted; however, they had considerable amounts of precipitating antibody which was detectable earlier than in animals immunized with low antigen doses.

AgD itself was immunogenic when administered at 3 week intervals in adjuvants (Table II). IgG_1 and IgE titers were first detectable one week after the second immunization. Immunogenicity was related to dose and adjuvant. Titers were detected earliest if AgD was given on alum although maximum titers were similar with CFA and alum after three or four immunizations. AgD was least immunogenic in alginate. Precipitating antibodies were never detected in serum from mice immunized with AgD.

Because of the immunogenicity of AgD, it could only be used at low doses for in vivo suppression studies. Administration of 50 or 100μg of AgD on alum or 100μg in CFA, alginate, or saline did not result in suppression of homocytotropic antibody responses when the

Table I: Maximum IgG_1 and IgE titers obtained with different immunization regimes. LAF_1 mice were immunized every 21 days with 1 or 10μg of purified Ascaris, AgB-Asc, or WST on alum or 250μg of WST in CFA. Three and 48 hour PCA titers were measured 7 days following the third immunization by challenge with 100μg of WST.

TABLE I

Maximum IgE and IgG_1 Titers

immunogen/adjuvant	IgG_1 Titer[1]	IgE Titer
1μg Asc/alum	800	1600
10μg Asc/alum	1200	600
1μg AgB-Asc/alum	600	1600
10μg AgB-Asc/alum	300	300
1μg WST/alum	1200	1200
10μg WST/alum	1600	800
250μg WST/CFA	20	40

1. reciprocal of the highest dilution giving a 5X5mm PCA reaction in three test animals.

Table II: Antigen D immunogenicity. LAF_1 mice were immunized every three weeks with various doses of AgD in adjuvant. Serum pools obtained one week following the second, third, and fourth immunizations were titered by homologous PCA; test animals were challenged at 3 or 48 hours with 100μg of WST. Antibody responses to 10μg of WST on alum are shown for comparison.

TABLE II

Antigen D Immunogenicity

immunizing antigen/adjuvant	two[1]		three		four	
	IgG_1[2]	IgE	IgG_1	IgE	IgG_1	IgE
100μg AgD/alum	<10	20	200	400	100	200
500μg AgD/alum	50	100	800	200	800	400
100μg AgD/alginate	-[3]	-	-	-	-	-
500μg AgD/alginate	-	-	10	<10	100	800
1mg AgD/alginate	-	-	10	10	50	50
500μg AgD/CFA	-	-	100	400	400	400
1mg AgD/CFA	50	100	400	800	800	800
10μg WST/alum	400	800	1200	800	1600	800

1. number of immunizations
2. titer: reciprocal of the highest serum dilution giving a 5X5 mm PCA reaction in three test animals
3. no detectable antibody

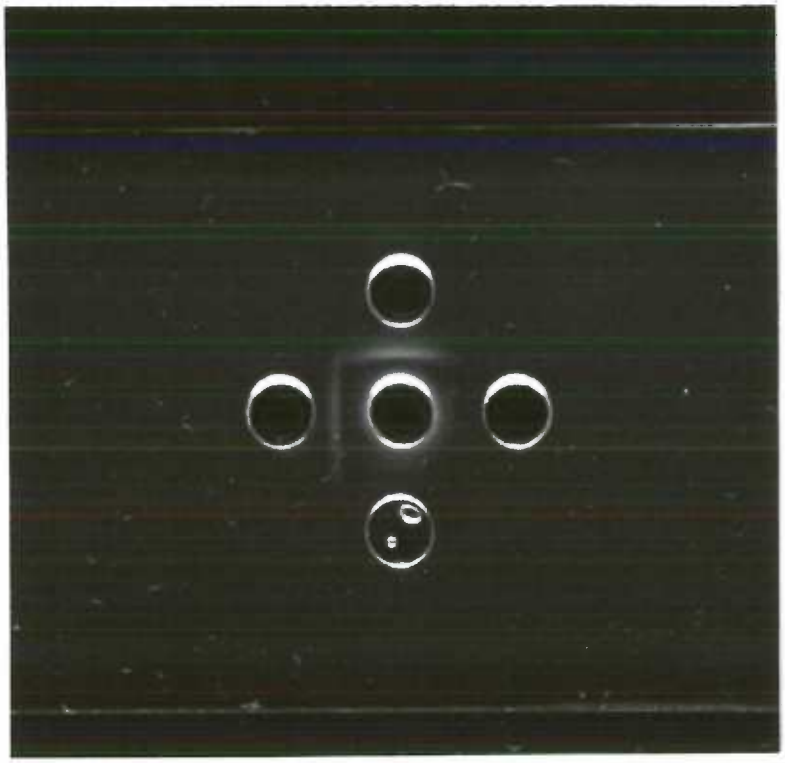
AgD was given with or 24 hours before the second, third, and fourth immunizations with 10μg of WST on alum. In vivo suppression was not pursued at this time because of the AgD immunogenicity and the difficulty of quantitating effective doses in adjuvant.

Antibody Specificities:

Specificities of the precipitating antibodies were determined by immunodiffusion. Sera were tested against purified antigen fractions; rabbit antisera against AgA+B or WST were used as references. Animals immunized with 1 or 10μg of WST on alum or 250μg of WST in CFA produced antibodies against AgA, and alum immunization sometimes induced small amounts of antibody to AgB. Other pollen determinants which have not been characterized were also recognized as demonstrated by two to three precipitin bands against WST (Figure 4). If serum was preincubated with AgD, there was no detectable inhibition of precipitation which was also indicative of the lack of specificity for AgB. Animals immunized with AgB-Asc had no detectable precipitating antibody against WST but had strong precipitin lines against Ascaris.

Specificities of homocytotropic antibodies were determined by using purified components of timothy pollen for PCA challenge. The titers induced with optimum amounts of WST were compared with those induced with optimum amounts of AgB or AgA (Table III). In the experiment shown, two serum pools were used, one from LAF_1 mice immunized four times with 1μg of AgB-Asc and the other from C3H mice immunized twice with 10μg of WST. As expected, practically all anti-timothy

Figure 4: Specificity of precipitating antibody. LAF_1 serum was obtained one week after a fourth immunization with 10μg of WST on alum and placed in the center well. Outer wells (clockwise from top) contained 1mg protein WST/ml, 1mg protein AgB/ml, 0.5mg protein WST/ml, and 1mg protein AgA/ml. The representational drawing is included to clarify the photograph.



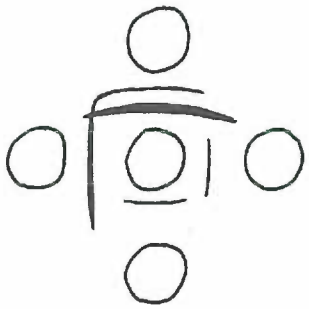


Table III: Specificities of homocytotropic antibodies. Serum pools from LAF_1 mice immunized four times with 1μg of AgB-Asc on alum or from C3H mice immunized twice with 10μg of WST on alum were titered by PCA. Antigens used for 3 and 48 hour challenge were WST, AgA, and AgB.

TABLE III

Specificities of Homocytotropic Antibodies

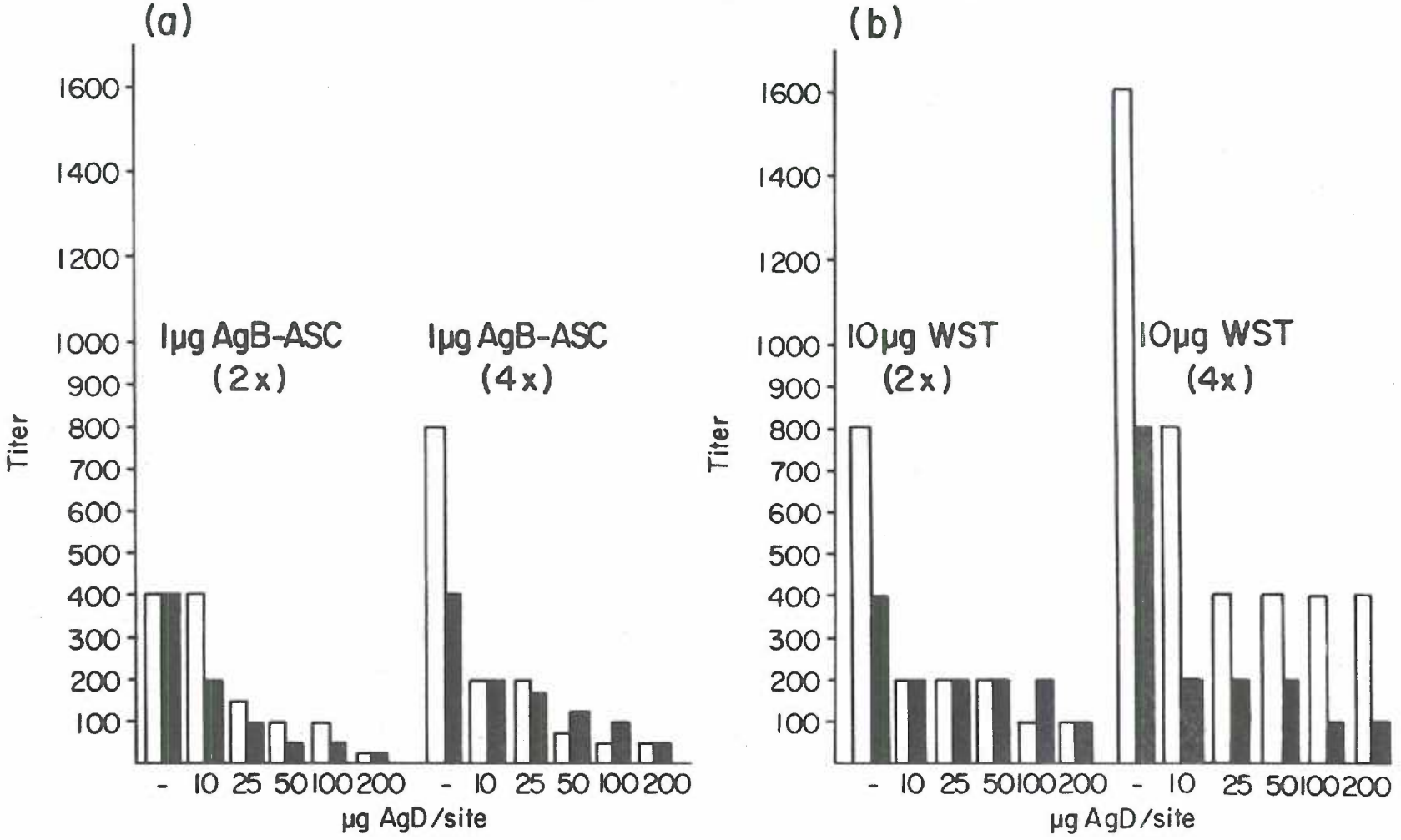
mouse strain	immunogen/ adjuvant	challenge antigen	IgG_1[1] titer	IgE titer
LAF_1	1μg AgB-Asc/alum	100μg WST	800	400
		50μg AgB	800	400
		50μg AgA	50	100
C3H/HeJ	10μg WST/alum	100μg WST	100	100
		50μg AgB	80	50
		50μg AgA	0	<5

1. reciprocal of the highest serum dilution giving a 5X5mm PCA reaction in three test animals

antibody in the first group was directed against AgB, the immunogen. Titers using WST and AgB for challenge were equivalent. The low titers induced with AgA probably reflect the slight degree of cross reactivity between antigens A and B. Of particular interest was the observation that AgB was the major determinant recognized even when the crude pollen extract was used for immunization. Since AgD is monovalent for the AgB determinant, this allowed me to conduct AgD inhibition studies to confirm the restricted antigenic specificity of the homocytotropic antibodies.

Inhibition studies were designed in two ways. First, various amounts of AgD, ranging from 50 to 500μg per mouse, were injected intravenously into serum implanted Swiss Webster mice 10 to 30 minutes before challenge with 25 or 100μg of WST. With doses of equal to or greater than 250μg, AgD itself induced PCA reactions, and with lower doses no inhibition was demonstrable. However, in a second series of experiments, a dose dependent AgD inhibition of both IgG_1 and IgE antibodies occurred if the AgD was preincubated with serum before PCA assay (Figure 5). In this system a threshhold amount of WST (25μg) was used for challenge to maximize the detection of inhibition. AgD excess ranged from one to 25 fold over the WST challenge dose, and animals challenged with dye alone had no reactions, an indication that AgD itself did not cause histamine release. Therefore, a greater AgD excess could be obtained by serum preincubation than by intravenous pretreatment, and this could explain the discrepancy between the results obtained with these two methods. AgD inhibition was antigen specific since anti-Ascaris titers were not significantly

Figure 5: Antigen D inhibition of IgG_1 and IgE PCA activity. Dilutions of pooled serum from LAF_1 mice immunized 2 or 4 times with 1μg of AgB-Asc on alum (a) or 2 or 4 times with 10μg of WST on alum (b) were preincubated with various amounts of AgD before PCA assay. Mice were challenged at 3 or 48 hours with 25μg of WST. Open bars represent IgG_1 titers and solid bars represent IgE titers; titers are the reciprocals of the highest serum dilutions giving 5X5mm PCA reactions in three test animals.


(a)
1600
1400
1200
1000
900
800
700
600
500
400
300
200
100
Titer
1μg AgB-ASC
(2x)
1μg AgB-ASC
(4x)
- 10 25 50 100 200 - 10 25 50 100 200
μg AgD/site
(b)
1600
1400
1200
1000
900
800
700
600
500
400
300
200
100
Titer
10μg WST
(2x)
10μg WST
(4x)
- 10 25 50 100 200 - 10 25 50 100 200
μg AgD/site

decreased by AgD preincubation.

If the assumption is made that the specificity of serum antibodies reflects the specificity of the receptors on B cells producing those antibodies, then these experiments suggest that B cells synthesizing IgE and IgG_1 are specific for AgB and that B cells producing precipitating IgG and IgM are specific for AgA. There is also a minor population(s) of B cells producing precipitating antibodies directed against other undefined components of timothy pollen.

Definition of Lymphocyte Transformation - Response to Antigen:

As another parameter of the immune response to timothy pollen antigens, I developed a micro lymphocyte transformation assay system according to the method of Strong, Ahmed, Thurman, and Sell (1973). Several variables were examined in order to define optimum conditions for the assay.

Figure 6 shows the responses of mitogen and antigen stimulated cultures at 24 hour intervals after the initiation of culture. Mitogen responses were maximum at 48 to 72 hours; antigen responses were optimum at 96 or 120 hours depending on the antigen dose. For all subsequent experiments, mitogen stimulated cultures were harvested at 72 hours and antigen stimulated cultures at 120 hours.

Dose response studies were done with all mitogens and antigens. Figure 7 shows the 72 hour responses of normal LAF_1 spleen cells to increasing doses of Con A, PHA, and LPS. Optimum mitogen doses were 0.25 to 1.0μg Con A per culture, 0.025% PHA, and 0.5μg LPS per culture.

Figure 6: Lymphocyte transformation responses at various times after the initiation of culture. Normal LAF_1 spleen cells (a) were cultured with 1.0μg Con A (triangles), 0.05% PHA (open circles), or 0.5μg LPS (squares) and harvested at 24 hour intervals. Responses were calculated as % maximum response; the maximum response was obtained at 72 hours to Con A (221,650cpm).

Spleen cells from mice immunized twice with 10μg of WST on alum (b) were cultured with 0.1μg WST (triangles), 1.0μg WST (open circles), or 10μg WST (squares). Responses were calculated as % maximum response to WST (55,715cpm at 120 hours to 1.0μg WST).

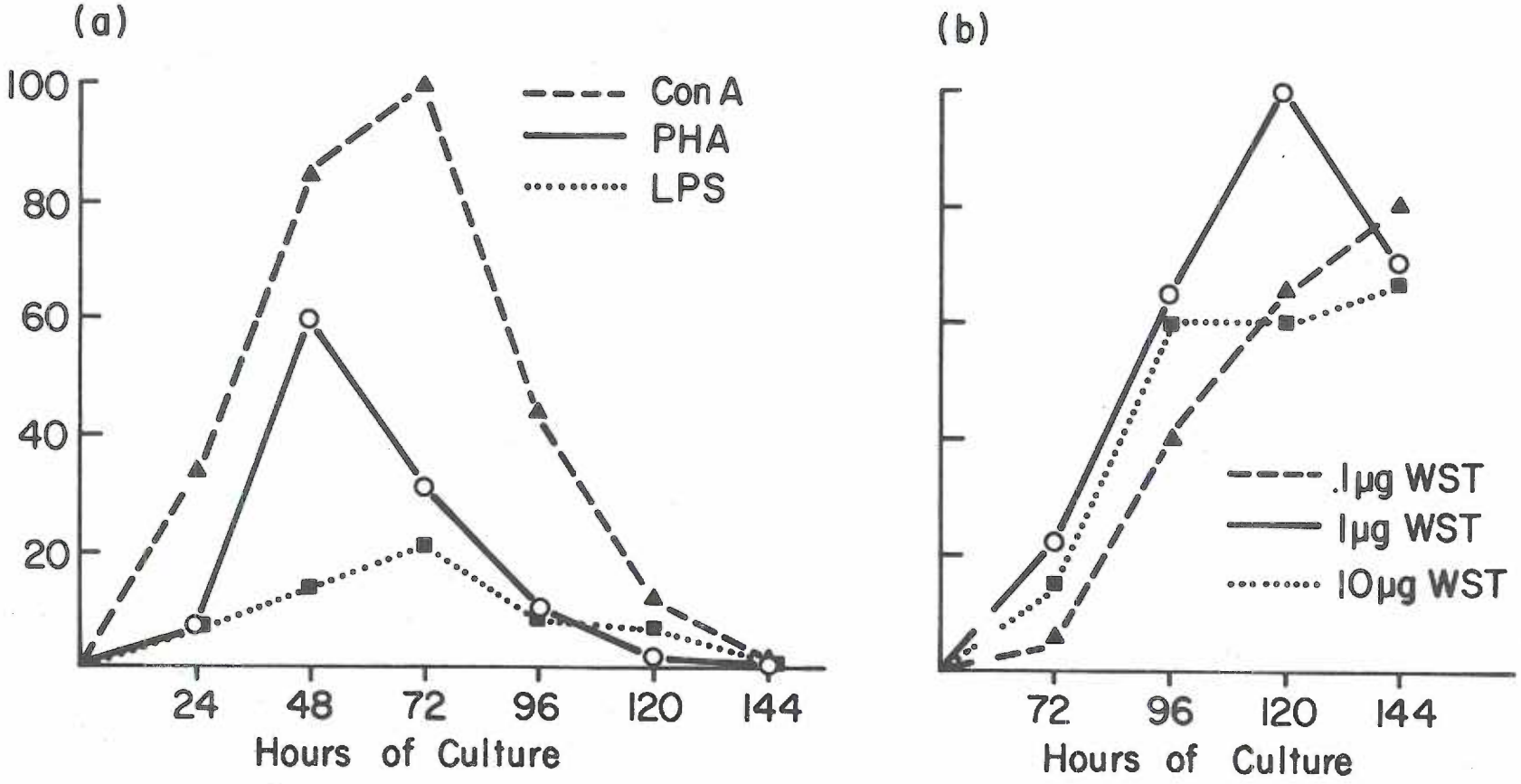
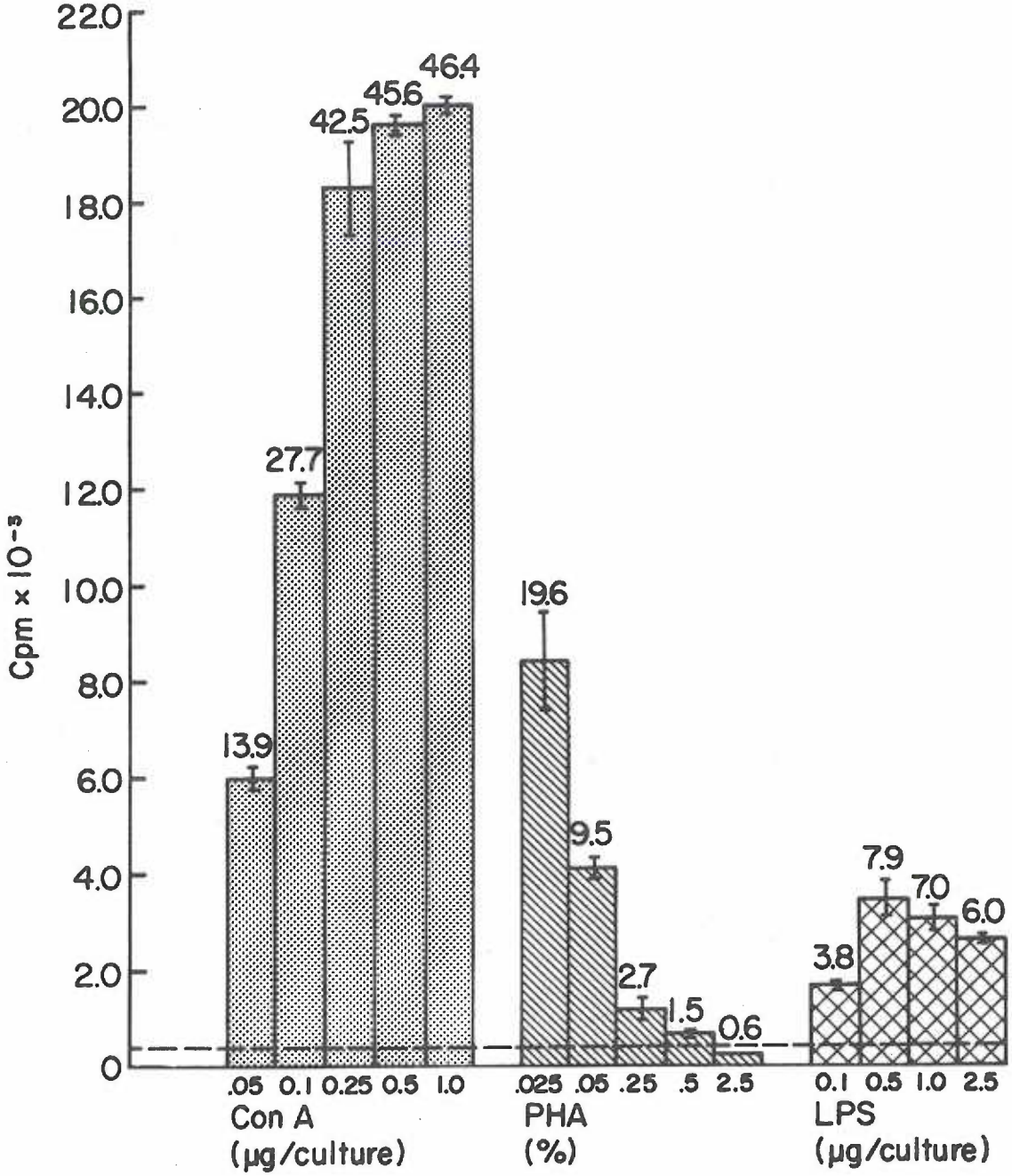

(a)
100
80
60
40
20
Con A
PHA
LPS
24
48
72
96
120
144
Hours of Culture
(b)
.1µg WST
1µg WST
10µg WST
72
96
120
144
Hours of Culture

Figure 7: Mitogen dose responses. Normal LAF_1 spleen cells were cultured for 72 hours with varying doses of Con A, PHA, or LPS. Responses are expressed as average cpm/culture ± standard error. Cell background is indicated by the dashed line, and stimulation indices are given above each column.

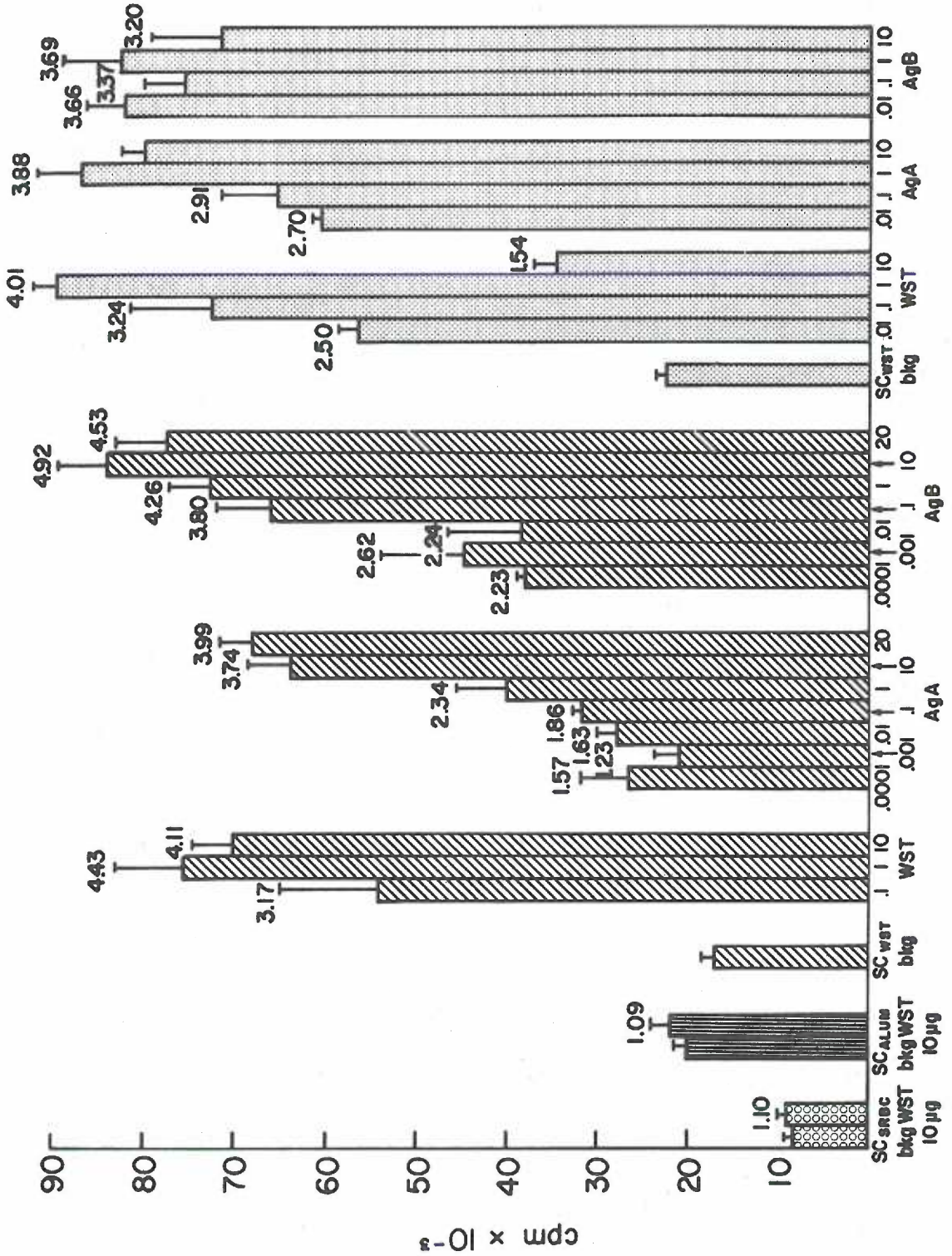

22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
Cpm x 10⁻³
13.9
27.7
42.5
45.6
46.4
19.6
9.5
2.7
1.5
0.6
3.8
7.9
7.0
6.0
.05 0.1 0.25 0.5 1.0
Con A
(µg/culture)
.025 .05 .25 .5 2.5
PHA
(%)
0.1 0.5 1.0 2.5
LPS
(µg/culture)

In subsequent experiments, doses of less than 0.025% PHA or greater than 1.0μg Con A per culture were tested and resulted in lower stimulation than the optimum concentrations listed above. Stimulation indices varied in different experiments, the averages being 29 (range 7.5 to >100) for Con A, 11 (range 4 to 30) for PHA, and 5 (range 2 to 15) for LPS.

Dose response curves with spleen cells from two groups of LAF_1 mice immunized twice with 10μg of WST on alum and stimulated in culture with WST, AgA, or AgB are shown in Figure 8. Spleen cells from animals immunized with alum only or animals immunized twice with $5X10^8$ SRBC did not respond to WST. Spleen cells from SRBC immunized mice could be stimulated maximally in vitro with $5X10^5$ SRBC (stimulation index of 2.3 to 4.9), but this same dose of SRBC did not stimulate normal or WST immune spleen cells (not shown).

As expected, spleen cells from mice immunized with crude extract responded to all antigen fractions in vitro. Dose response curves for all timothy pollen antigens were very broad, in some experiments extending from 0.0001 to 20μg per culture. When comparing experiments, it was found that sometimes one antigen fraction was more stimulatory than another, but I have been unable to correlate these variations with differences in the immune status of the spleen cell donors; they are not related to number of immunizations or time between the last immunization and sacrifice. Furthermore, no consistent differences in the dose response curves have been seen to indicate changes in receptor avidities with different numbers of immunizations or time after boosting.

Figure 8: Antigen dose responses. Spleen cells from LAF_1 mice immunized twice with SRBC (SC_{SRBC}), alum (SC_{alum}), or 10μg WST on alum (SC_{WST}; two experiments) were cultured with various doses of WST, AgA, and AgB and harvested at 120 hours. SC_{SRBC} and SC_{alum} were cultured with 10μg of WST only. Responses were calculated as average cpm/culture ± standard error, and stimulation indices are indicated above each column.


cpm x 10⁻³
90
80
70
60
50
40
30
20
10
SC SRBC bkg WST 10 µg
SC ALLO bkg WST 10 µg
SC WST bkg
WST .1 1 10
AgA .0001 .001 .01 .1 1 10 20
AgB .0001 .001 .01 .1 1 10 20
SC WST bkg
WST .01 .1 1 10
AgA .01 .1 1 10
AgB .01 .1 1 10
1.10
1.09
4.43
4.11
3.17
1.57
1.86
1.63
1.23
2.34
3.99
3.74
2.23
2.24
2.62
4.92
4.53
4.26
3.80
2.50
3.24
4.01
1.54
2.70
2.91
3.88
3.66
3.37
3.69
3.20

Figures 9 and 10 illustrate the changes in the response to WST with time after a primary and secondary immunization with 10μg of WST on alum (Figure 9) or 250μg of WST in CFA (Figure 10). Response patterns in the two groups of mice were similar. Maximum responses to antigen occurred 6 to 10 days after a primary immunization and 5 to 6 days after a boost; times of significant response were relatively narrow, having declined to background by day 12 after a primary and day 10 after a secondary immunization. Consequently, all subsequent assays were performed 3 to 8 days after boosting. Day 5 proved to be the least advantageous time for assay because background responses showed a similar pattern of increase after immunization and peaked at day 5; therefore, stimulation indices at this time were often low.

Figures 9 and 10 also plot the IgG_1 and IgE titers. The homocytotropic antibody responses were very low after the primary immunization, and were boosted only in the group immunized with 10μg of WST on alum. Precipitating antibody was not detectable after the primary immunization. After a boost, all antibody titers peaked slightly later than the spleen cell responses and remained elevated slightly longer. Because of the similarities in lymphocyte transformation responses after primary and secondary immunizations and in different groups of mice compared with the differences in antibody responses, it can be concluded that the magnitude of the in vitro response to antigen is not related to the magnitude of the antibody response. However, there is a secondary in vitro response since the time of peak reactivity occurs earlier after a boost than after the primary

Figure 9: Lymphocyte transformation and antibody responses at different times after primary and secondary immunizations with 10μg of WST on alum. Background (solid circles) and responses to 1μg (squares) or 10μg (open circles) of WST were measured at various times after immunization and are presented as average cpm/culture ± standard error. IgG_1 (open circles) and IgE (closed circles) titers were also determined and are represented in the bottom half of the figure; titers represent the highest serum dilutions giving 5X5mm PCA reactions in three test animals.

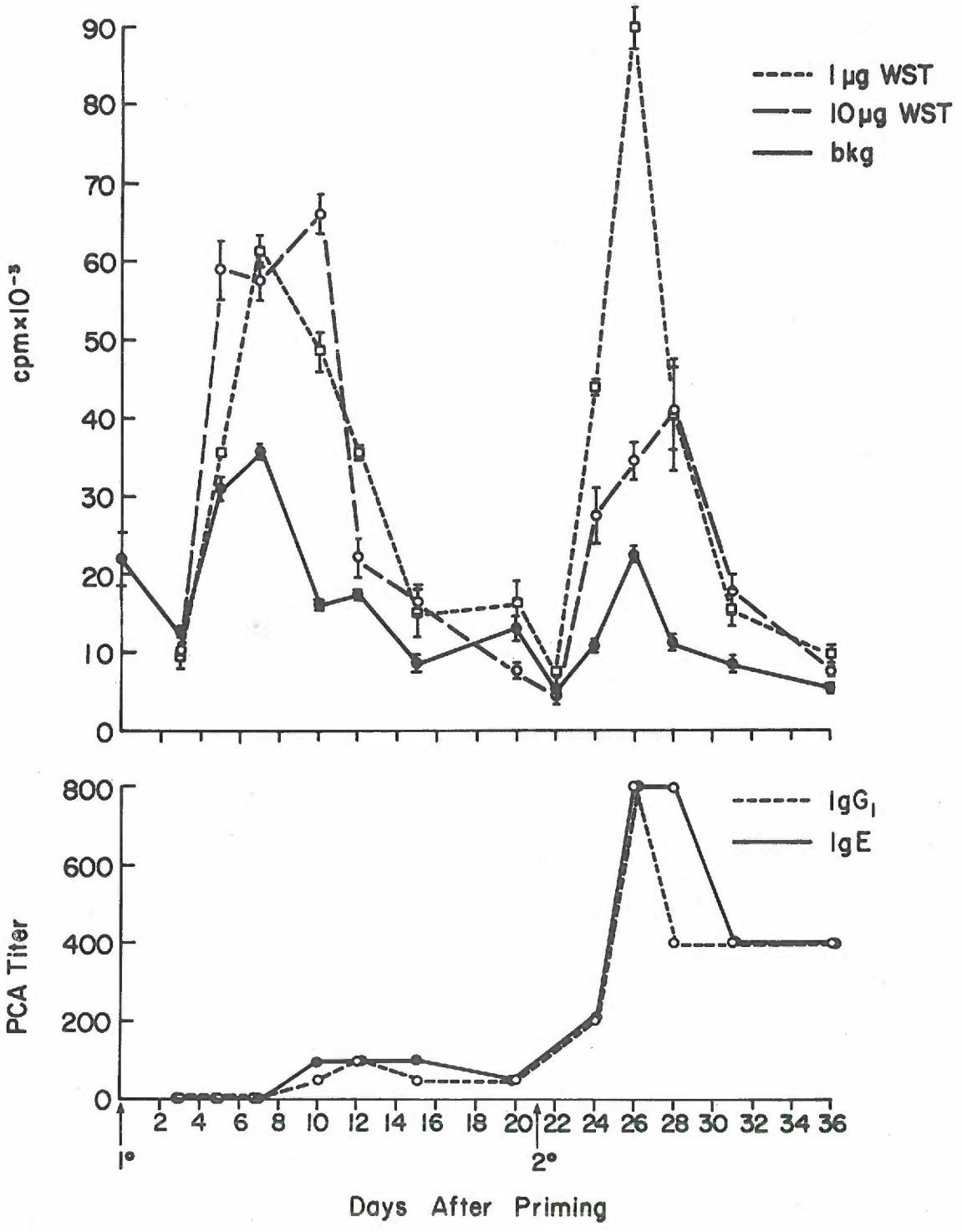
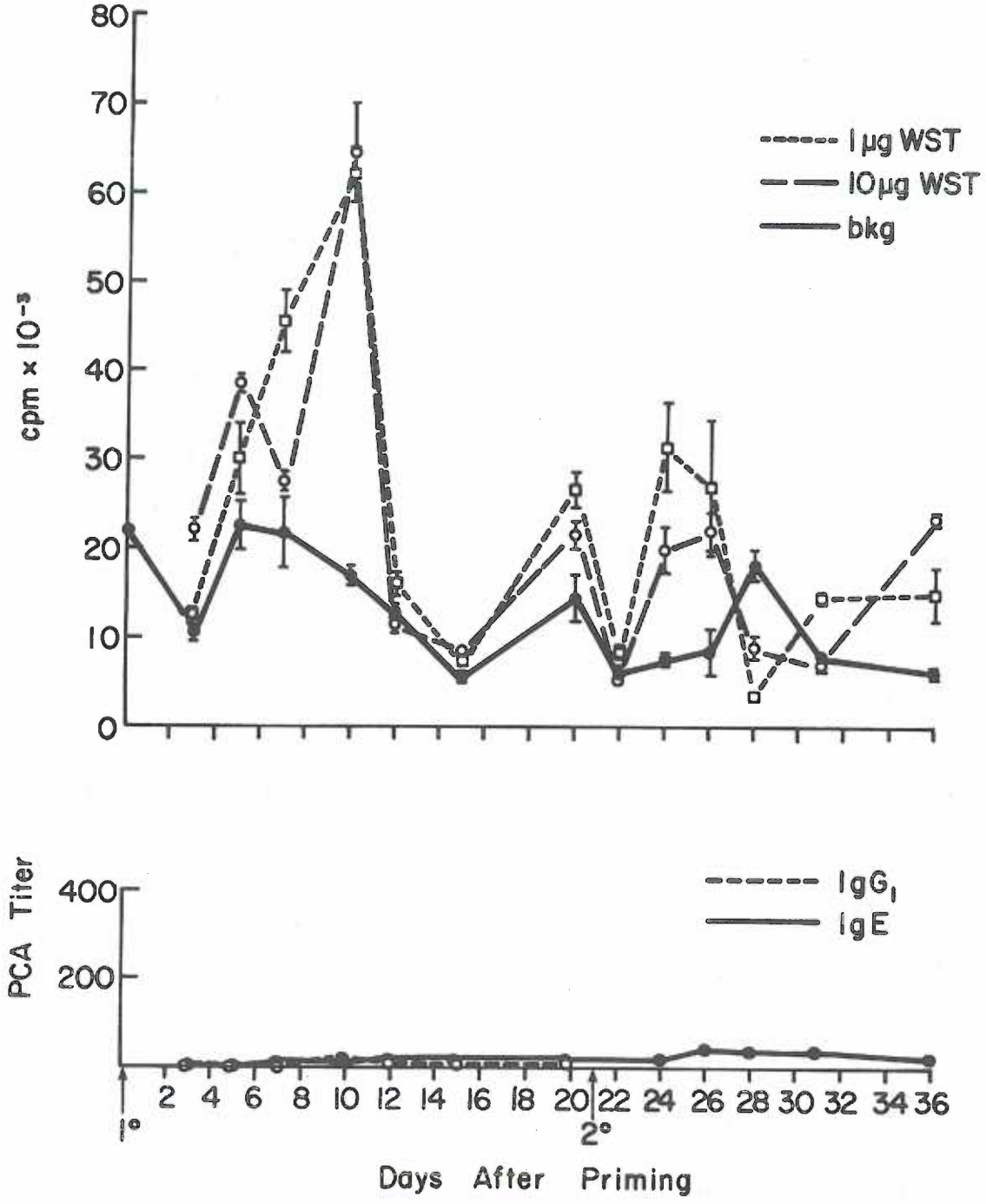

cpm×10⁻³
90
80
70
60
50
40
30
20
10
0
1 µg WST
10 µg WST
bkg
PCA Titer
800
600
400
200
0
IgG1
IgE
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
1°
2°
Days After Priming

Figure 10: Lymphocyte transformation and antibody responses at different times after primary and secondary immunizations with 250μg of WST in CFA. Background (solid circles) and responses to 1μg (squares) or 10μg (open circles) of WST were measured at various times after immunization and are presented as average cpm/culture ± standard error. IgG_1 (open circles) and IgE (closed circles) titers were also determined and are represented in the bottom half of the figure; titers represent the highest serum dilutions giving 5X5mm PCA reactions in three test animals.


80
70
60
50
40
30
20
10
0
cpm x 10⁻³
1 µg WST
10µg WST
bkg
PCA Titer
400
200
IgG1
IgE
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
1°
2°
Days After Priming

immunization.

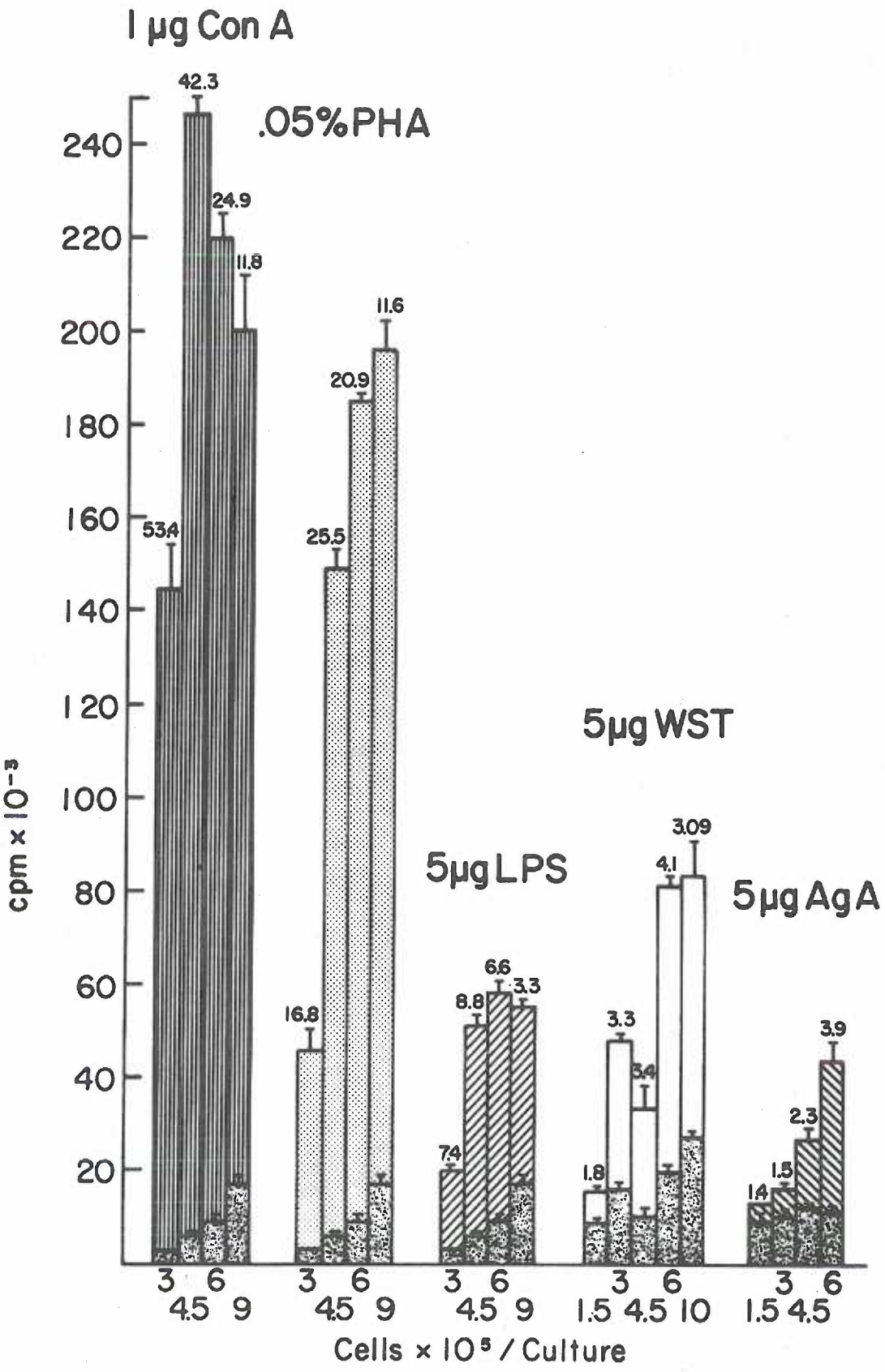
Responses to Con A, PHA, and LPS (not shown) demonstrated an inverse type of response pattern; thymidine uptake was decreased 50 to 100% at the times of peak response to antigen and began to recover 10 to 12 days after immunization. Since both T and B cell mitogen responses were decreased, this seems to be a general anergy to non-specific stimuli.

The effect of cell number on the maximum in vitro response was the last variable of the lymphocyte transformation system examined. Figure 11 shows the effect of varying the number of cells per culture on the mitogen and antigen responses. For all stimulants, 4.5 to $6X10^5$ cells per culture was optimum. Although not shown, optimum mitogen and antigen doses were the same over the entire range of cell concentrations tested. Unless otherwise stated, I have consistently cultured $6X10^5$ cells.

Antigen D Inhibition in Vitro:

Having determined that spleen cells from WST immune mice respond to all timothy pollen antigens, I next questioned whether the responses could be inhibited by AgD. Since AgD has the AgB determinant, I would expect at least partial inhibition of the responses to AgB and WST if B cells are proliferating to antigen in culture. On the other hand, I would expect no inhibition of the B cell response to AgA. The carrier specificity of AgD has not been defined, but the observation that it is weakly immunogenic in mice suggests it reacts with T cells

Figure 11: Responses of various numbers of spleen cells. Normal cells at 3 to $9X10^5$ cells per culture were stimulated with Con A, PHA, or LPS and harvested at 72 hours. Spleen cells from mice immunized twice with 10μg of WST on alum were cultured at 1.5 to $10X10^5$ cells per culture with 5μg WST or 5μg AgA and harvested at 120 hours. Average cpm/culture ± standard error and stimulation indices were calculated for all cultures.


1 µg Con A
.05% PHA
5µg WST
5µg LPS
5µg AgA
cpm x 10⁻³
240
220
200
180
160
140
120
100
80
60
40
20
42.3
24.9
11.8
53.4
25.5
20.9
11.6
16.8
8.8
6.6
3.3
7.4
3.09
4.1
3.3
3.4
1.8
3.9
2.3
1.5
1.4
3 6
4.5 9
3 6
4.5 9
3 6
4.5 9
3 6
1.5 4.5 10
3 6
1.5 4.5
Cells x 10⁵ / Culture

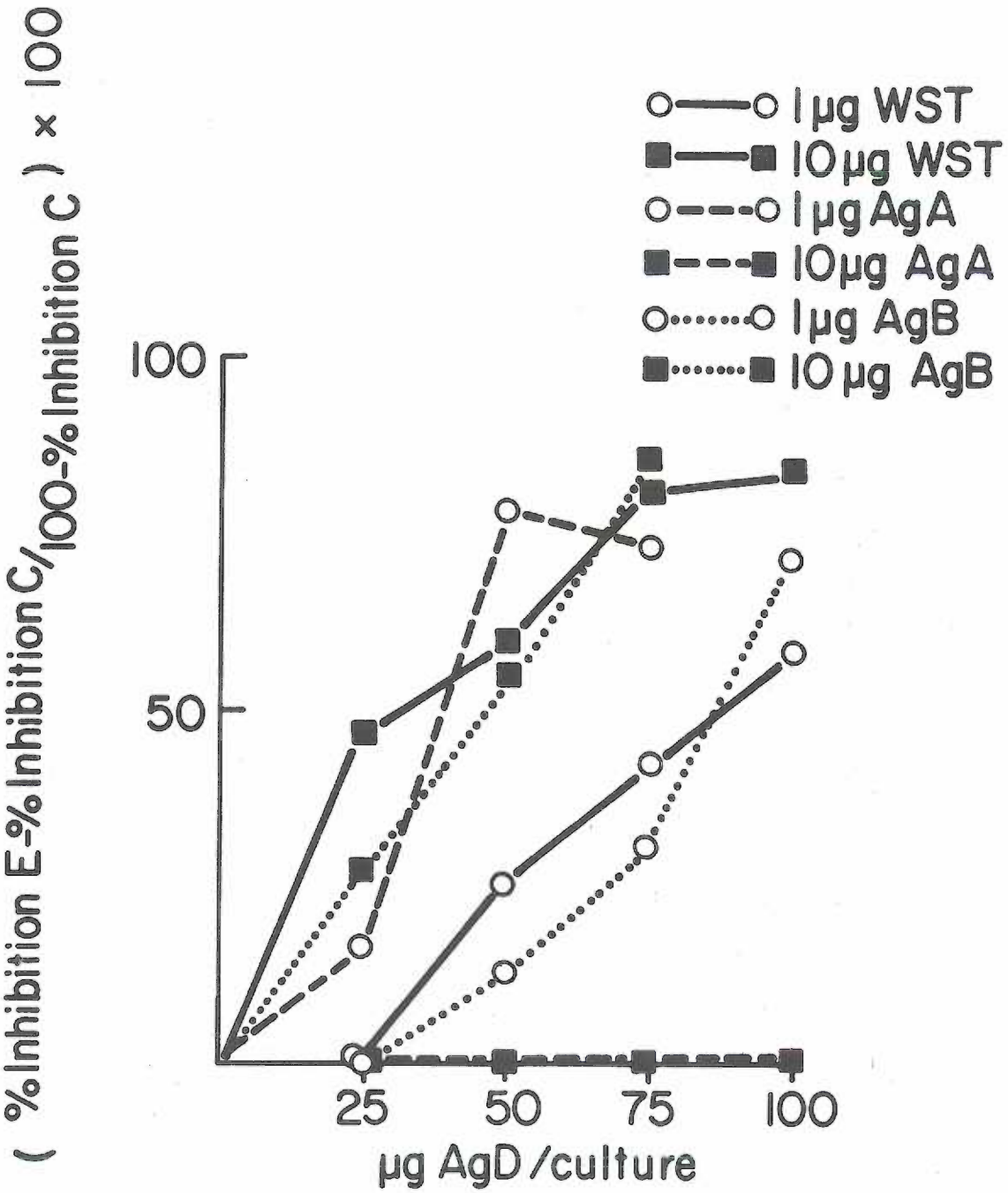
and might also be able to inhibit their response in vitro. Therefore, if T cells are proliferating in culture, AgD inhibition may help to define any heterogeneity of carrier determinants.

Control cultures showed that AgD is not stimulatory to normal or immune cells over a dose range of 0.001 ot 250μg per culture, that AgD does not inhibit responses to Con A, PHA, or LPS, and that AgD does not inhibit the response of SRBC-immune spleen cells to SRBC.

When AgD was added to cultures containing spleen cells from mice immunized twice with 10μg of WST on alum, it was able to inhibit responses to 1 or 10μg of WST, 1 or 10μg of AgB, or 1μg of AgA in a dose dependent fashion (Figure 12). However, AgD was unable to inhibit the response to a higher dose (10μg) of AgA. This suggests that AgA has at least two antigenic determinants which induce cell proliferation and therefore there must be at least two responding populations. It cannot be determined from this experiment whether these populations are hapten specific, carrier specific, or both. However, since AgD does not cross react serologically with AgA, it can be predicted that the population not inhibited by AgD is a B cell population and that AgD inhibition with low doses of AgA is due to a carrier determinant common to AgA and AgD.

Two general inhibition curves are seen in Figure 12. Responses to high doses of WST and AgB were inhibited by lower doses of AgD than responses to low doses of WST and AgB; 50% inhibition was obtained with about 38μg and 85μg of AgD for the two groups. Although not shown, 50% inhibition of the responses to higher doses of WST was also

Figure 12: Antigen D inhibition of lymphocyte transformation. Spleen cells from LAF_1 mice immunized twice with 10μg of WST on alum were cultured with various concentrations of AgD in the presence of 1μg (o———o) of 10μg (■———■) of WST, 1μg (o— —o) or 10μg (■— —■) of AgA, or 1μg (o·····o) or 10μg (■·····■) of AgB. Inhibition of responses to antigen was calculated after 120 hours of culture.

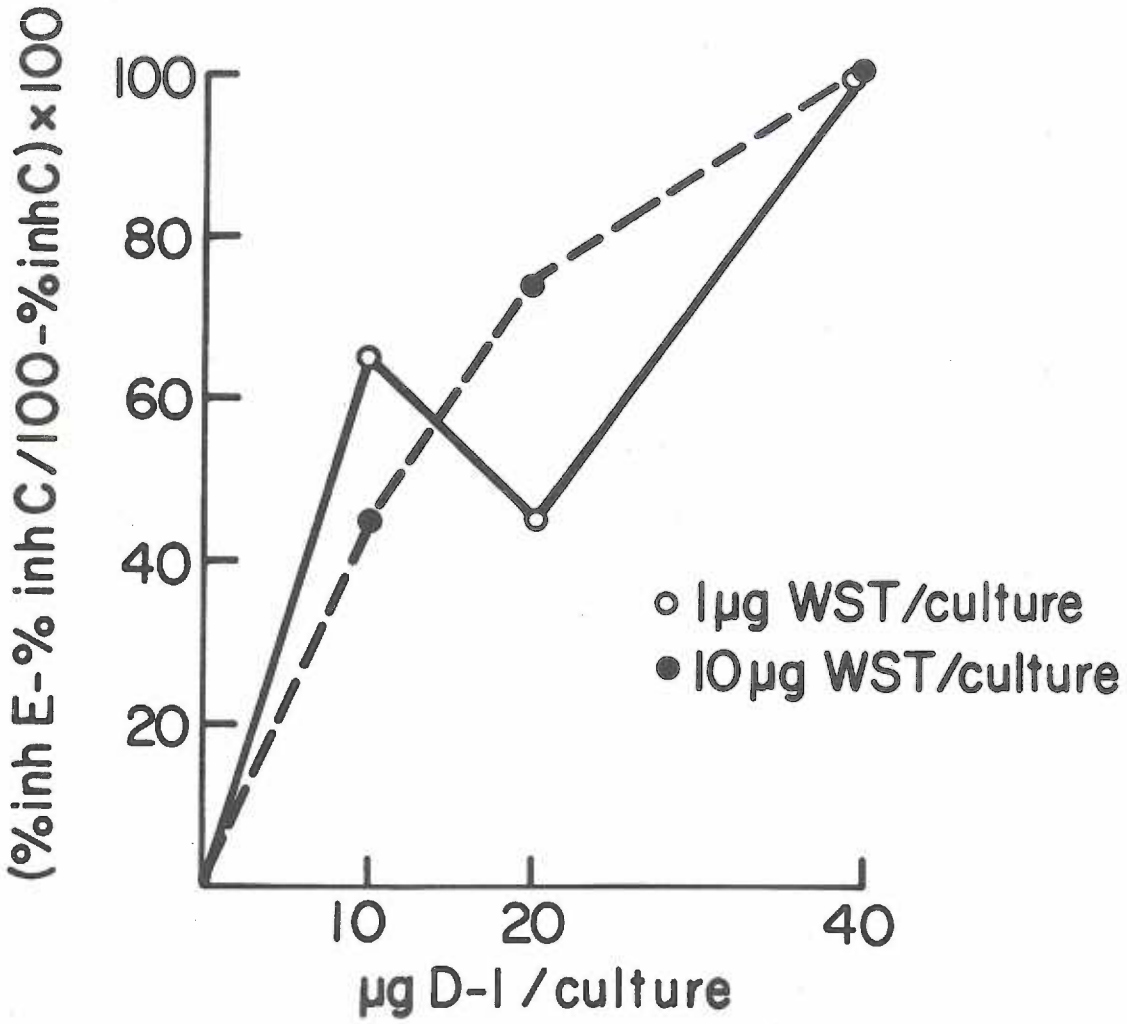

(%Inhibition E-%Inhibition C/100-%Inhibition C) × 100
100
50
25
50
75
100
µg AgD/culture
1 µg WST
10 µg WST
1 µg AgA
10 µg AgA
1 µg AgB
10 µg AgB

obtained with 35 to 40μg of AgD. These results implicate heterogeneity in the cell populations responding to WST and AgB as well as in the population responding to AgA. If these two curves were simply due to heterogeneity of receptor avidities, I would expect AgD inhibition to be more effective with low antigen doses. Since the reverse is true, it suggests that there is more than one population of cells responding to different antigenic determinants. This again could reflect responses to hapten and carrier determinants by B and T cells respectively; moreover, since AgB is the major antigenic component in the crude extract, the same cell populations could be responding to WST and AgB.

To try and dissociate hapten specific from carrier specific inhibition, Bio-gel P-2 fractions of AgD were tested for their abilities to inhibit lymphocyte transformation. All three fractions have the hapten determinant, but because of size restrictions it seems unlikely that they all have a carrier determinant as well. Due to a limited quantity of these fractions, only inhibition of the response to WST was tested.

Results from one of two inhibition experiments using D-1 (5,000 molecular weight) are illustrated in Figure 13. D-1 was able to inhibit the response to WST in a dose-dependent fashion. This was not surprising since D-1 is the largest fraction from AgD, and the inhibitory activity of the unfractionated AgD may be due to this species. D-2 (2,500 molecular weight) and D-3 (800 molecular weight) have also been tested. D-2 was highly toxic in cultures in a dose range of 1.0 to 100μg per culture and was not inhibitory at lower concentrations;

Figure 13: Inhibition of lymphocyte transformation by D-1. Spleen cells from LAF_1 mice immunized twice with 10μg of WST on alum were incubated with various doses of D-1 in the presence of 1μg (open circles) or 10μg (closed circles) of WST. Inhibition was calculated after 120 hours in culture.


(%inh E-% inh C/100-%inh C) x 100
100
80
60
40
20
10
20
40
µg D-1 / culture
o 1µg WST/culture
• 10µg WST/culture

D-3 was not inhibitory over a comparable dose range. Therefore, D-3 may lack a carrier determinant important for inhibition of lymphocyte transformation to WST. Yet, it does have a hapten determinant and I expected at least partial inhibition. It cannot be ruled out that the apparent noninhibitory effects of D-3 are due to low avidity binding to cell receptors.

Responses of Spleen Cells From Different Groups of Mice:

Another question I wished to examine was whether I could distinguish any differences in the responses of spleen cells from mice immunized with different antigens and/or adjuvants. Since IgE production can be induced in the absence of IgG production by immunization with AgB-Asc, and since IgE and IgG antibodies are directed against different determinants, I had hoped to be able to detect differences in hapten and carrier specific recognition events for IgE and IgG responses and possibly to answer the question of whether there is more than one population of helper T cells.

Three groups of mice were compared: first, mice immunized twice with 10μg of WST on alum to favor the production of homocytotropic antibodies; second, mice immunized twice with 1μg of AgB-Asc on alum to also favor the production of homocytotropic antibodies but of more restricted antigen specificity; and third, mice immunized twice with 250μg of WST in CFA to induce precipitating antibodies only. Sera from the first two groups had homologous PCA titers to AgB of 400 to 1600; the precipitating antibodies to pollen antigen were detectable

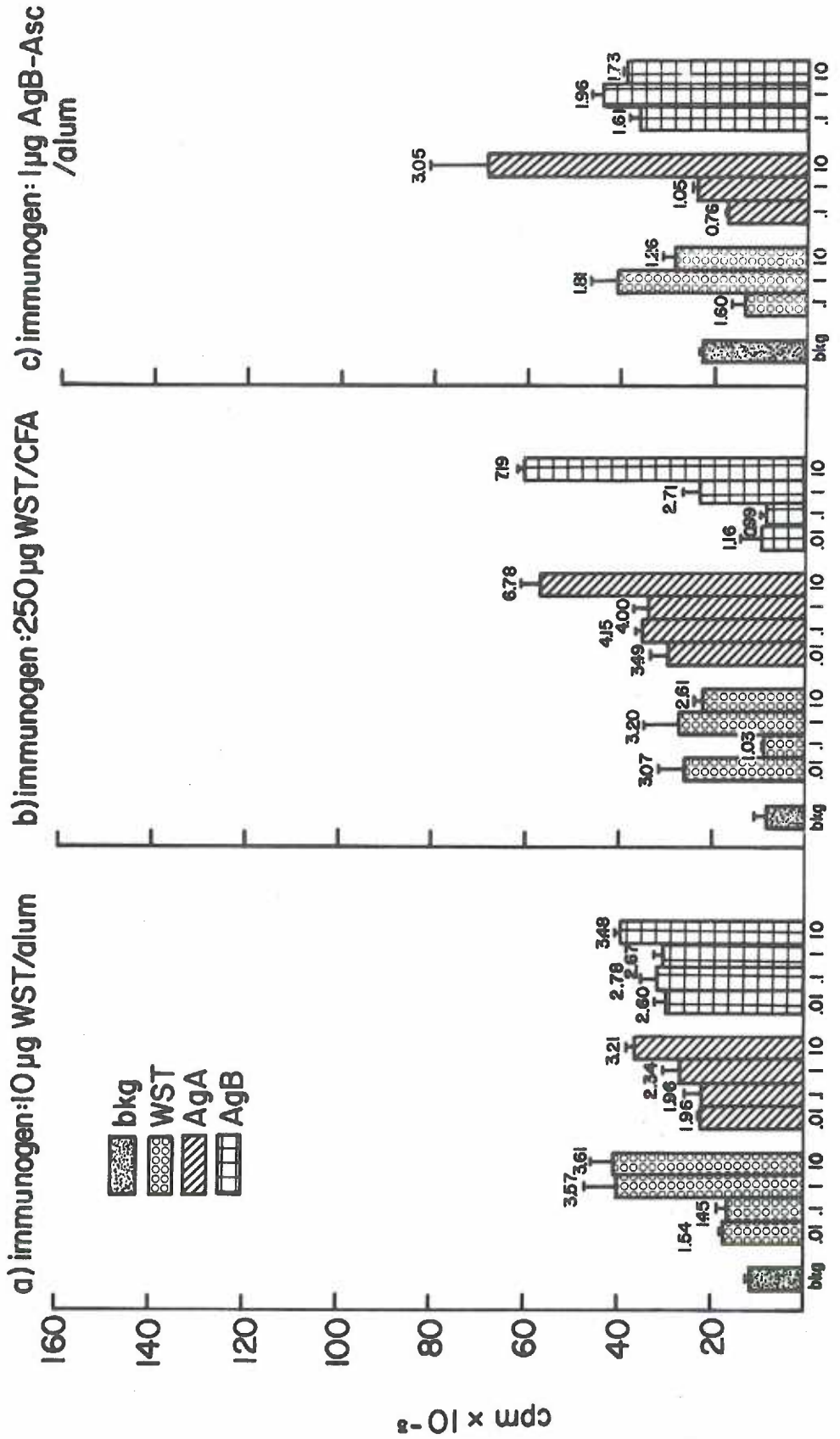
only in serum from mice immunized with crude extract since AgB appears to be a poor immunogen for the induction of IgM and IgG. Serum from the third group had significant precipitating antibody directed against AgA and very low levels of homocytotropic antibody (titers <10).

It might be expected that spleen cells from group one would respond to all antigen fractions, and that spleen cells from group two would respond to AgB and WST but to AgA only if there is a common carrier determinant and if T cells are proliferating in vitro. Spleen cells from group three might be expected to react strongly to AgA and WST and only weakly to AgB unless it shares a carrier determinant. Differences in dose response curves and maximum responses might also be anticipated since these groups have cells involved in production of different types and different amounts of serum antibody.

Dose response studies with all antigen fractions were performed with spleen cells from these three groups of mice, and representative results are presented in Figure 14. There were no clear cut differences in response patterns between the three groups; optimum antigen doses and maximum responses were similar. Since AgA induced lymphocyte transformation in AgB-Asc primed cells, either the small amount of the AgB determinant in AgA is inducing B cell stimulation or AgB acts as a carrier in vivo even after conjugation to Ascaris and primes a population of T lymphocytes. If the latter is true, carrier determinants on AgA and AgB must be identical.

Antigen D inhibition studies were also conducted with these three groups of mice, but due to limited quantities of AgD, inhibition of the responses to AgA and AgB were not evaluated. There were no

Figure 14: Antigen dose responses for three groups of mice. LAF_1 mice were immunized twice with 10μg of WST on alum (a), 250μg of WST in CFA (b), or 1μg of AgB-Asc on alum (c) and cultured for 120 hours with various doses of WST, AgA, or AgB. Average cpm/culture ± standard error and stimulation indices are plotted.


a) immunogen: 10 µg WST/alum
b) immunogen: 250 µg WST/CFA
c) immunogen: 1 µg AgB-Asc /alum
bkg
WST
AgA
AgB
cpm x 10⁻³
160
140
120
100
80
60
40
20
bkg .01 .1 1 10
1.54 1.45 3.57 3.61
1.96 1.96 2.34 3.21
2.60 2.78 2.67 3.48
1.16 .99 2.71 7.19
3.07 1.03 3.20 2.61
3.49 4.15 4.00 6.78
1.60 1.81 1.26 .76 1.05 3.05
1.61 1.96 1.73

consistent differences in the ability of AgD to inhibit responses to WST (Figure 15). In this particular experiment it appears as if the response of spleen cells from animals immunized with 250μg of WST in CFA was less susceptible to inhibition, but this tendency was not consistently observed when inhibition of responses to other doses of WST were measured. The two general inhibition profiles seen here are similar to those shown for high (10μg) and low (1μg) doses of WST (Figure 12). In the experiment of Figure 15 cells were cultured with 5μg of WST and different populations may respond differently to this intermediate dose.

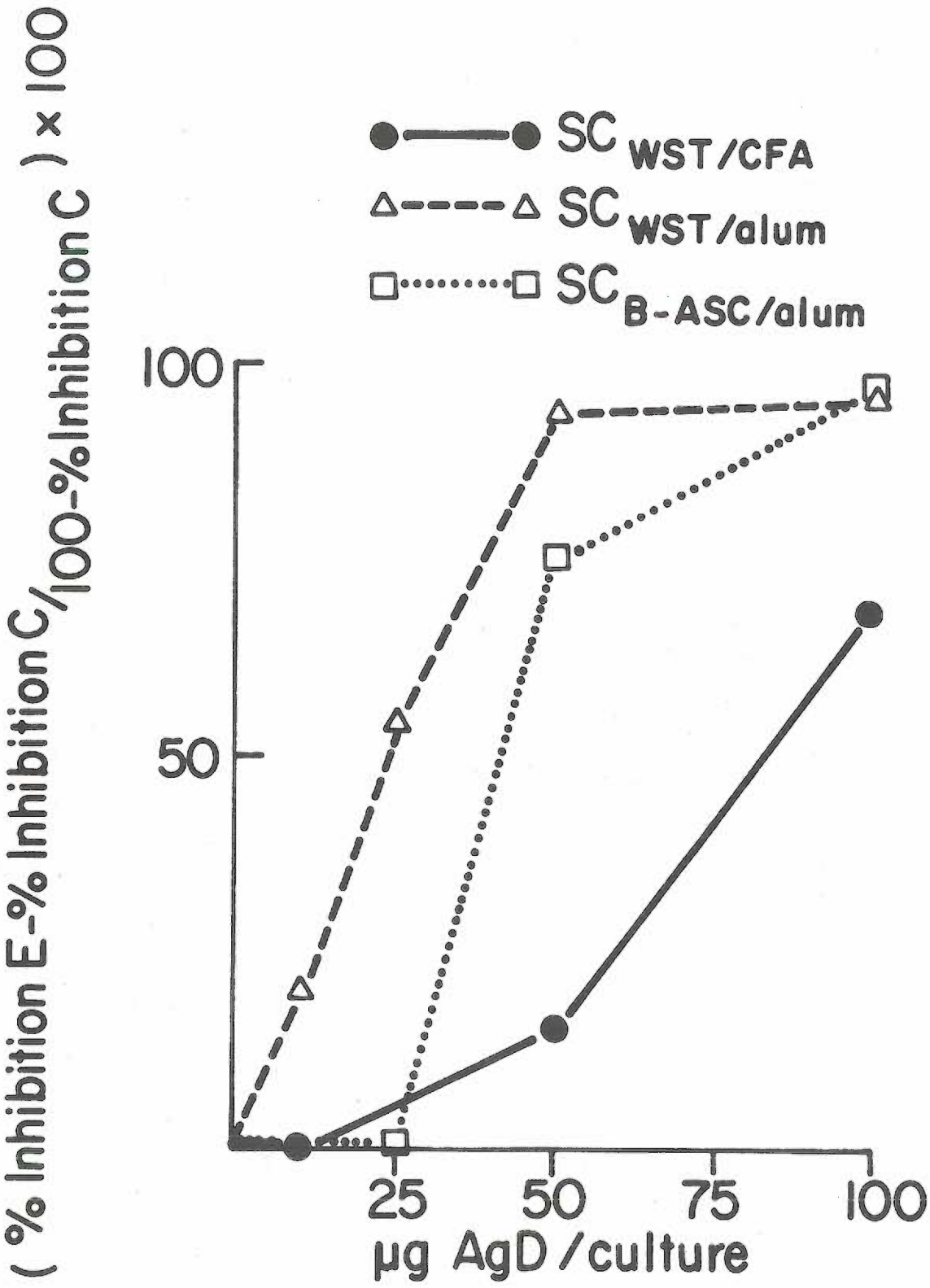
Isolation of B and T Lymphocytes:

To more carefully analyze hapten and carrier specific recognition events and the number of responding populations, B and T lymphocytes were isolated and cultured with purified pollen antigens in the presence or absence of AgD.

Optimum conditions for B and T cell isolation were first defined. Stable EA monolayers were obtained with 100μg/ml of poly-L-lysine, 1.5% SRBC, and a 1/150 dilution of RaSRBC. If lower antibody dilutions were used, the monolayers showed disruption following incubation with spleen cells. Dilutions of 1/150 and 1/300 gave similar results in terms of the recoveries and purities of the isolated populations; dilutions of greater than 1/300 resulted in decreased yields of B cells.

Several dilutions of spleen cells were also tried, and with

Figure 15: Antigen D inhibition of WST responses in three groups of mice. LAF_1 mice were immunized five times with 250μg of WST in CFA (circles), 10μg of WST on alum (triangles), or 1μg of AgB-Asc on alum (squares) and cultured for 120 hours with 5μg of WST in the presence of various amounts of AgD.


(% Inhibition E-% Inhibition C/100-% Inhibition C) x 100
SC WST/CFA
SC WST/alum
SC B-ASC/alum
100
50
25
50
75
100
µg AgD / culture

$6X10^6$ to $1.2X10^7$ cells/ml the relative recoveries and purities of the B and T cell populations were similar. With more cells, the percent of recovered cells in the unbound T cell population was increased as was the LPS response of this population; this suggested overloading of the monolayers. I have routinely used 10^7 cells/ml which approaches saturation. Of the cells applied, an average of 35% are recovered in the T cell population and 44% in the B cell population. If monolayers were coated with normal rabbit serum, >90% of the applied cells were recovered in the unbound fraction.

Purity of B and T cells isolated from the EA monolayers was evaluated by PHA and LPS stimulation. Figure 16 illustrates the responses of graded numbers of isolated B or T cells to PHA and LPS. Increasing numbers of B cells showed a linear increase in their response to LPS but not to PHA. T cells showed the opposite pattern; the response to PHA was high and increased proportionally with increasing numbers of cells whereas the LPS response remained low over the entire range of cells cultured. The responses of graded numbers of unfractionated spleen cells to PHA and LPS had slopes similar to those of the isolated populations but the curves were displaced to the right.

Figure 17 shows that when mixtures of B and T cells were cultured, the PHA response was again associated with the unbound or T cell fraction and the LPS response with the bound or B cell population. From these types of experiments, it has been calculated that the T cell population is contaminated with an average of 13% B cells and the B cell population with 8% T cells.

Enrichment of the populations has been further substantiated by

Figure 16: B and T cell responses to mitogens. Various numbers of normal B cells were cultured with 0.05% PHA (open triangles) or 0.5μg LPS (closed circles); normal T cells were also cultured with PHA (open circles) or LPS (closed squares). All cultures were harvested at 72 hours, and data are presented as average cpm/culture ± standard error; cell backgrounds have been subtracted. Responses of $6X10^5$ unfractionated normal spleen cells to PHA and LPS are represented by the individual high and low points respectively.

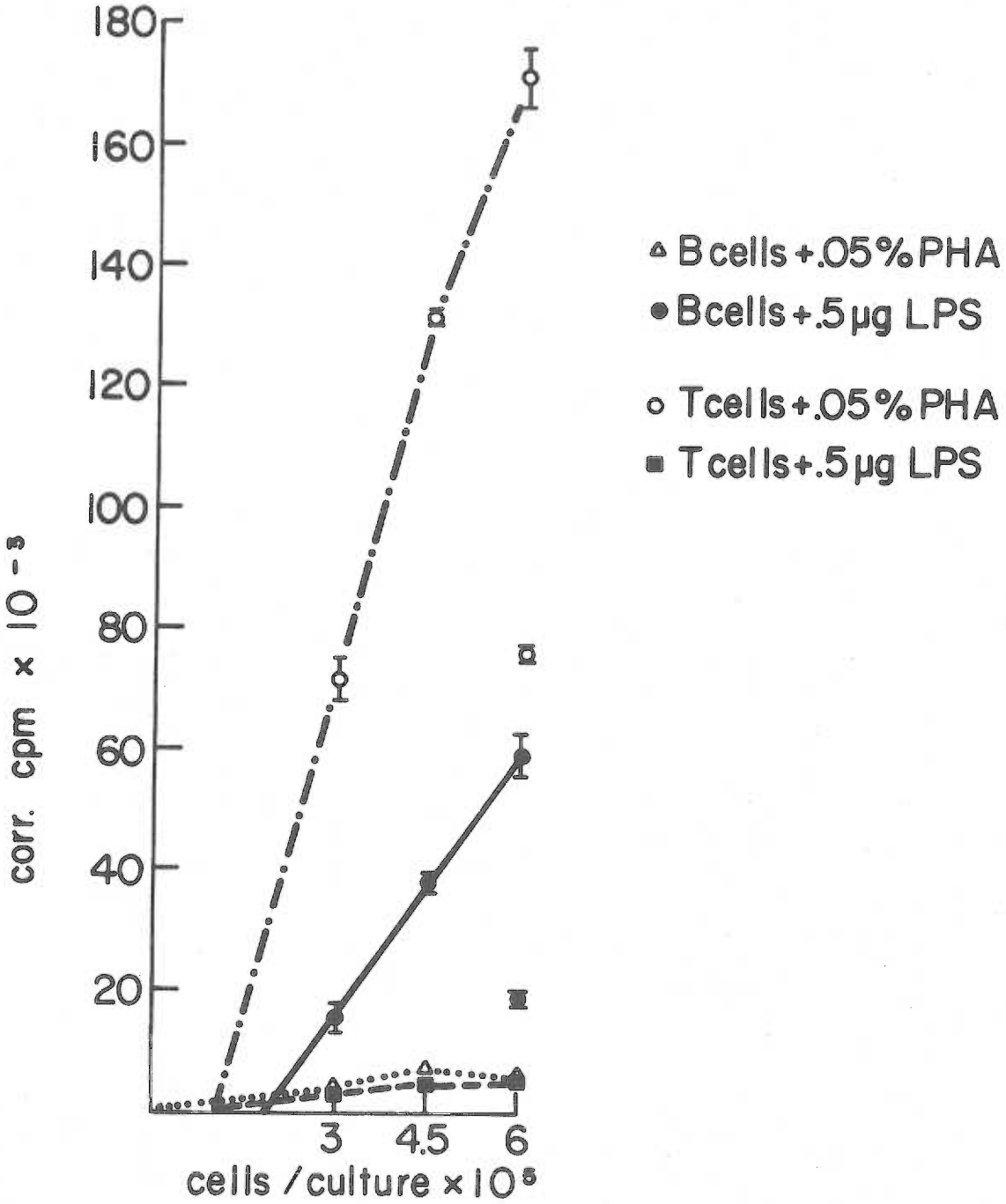
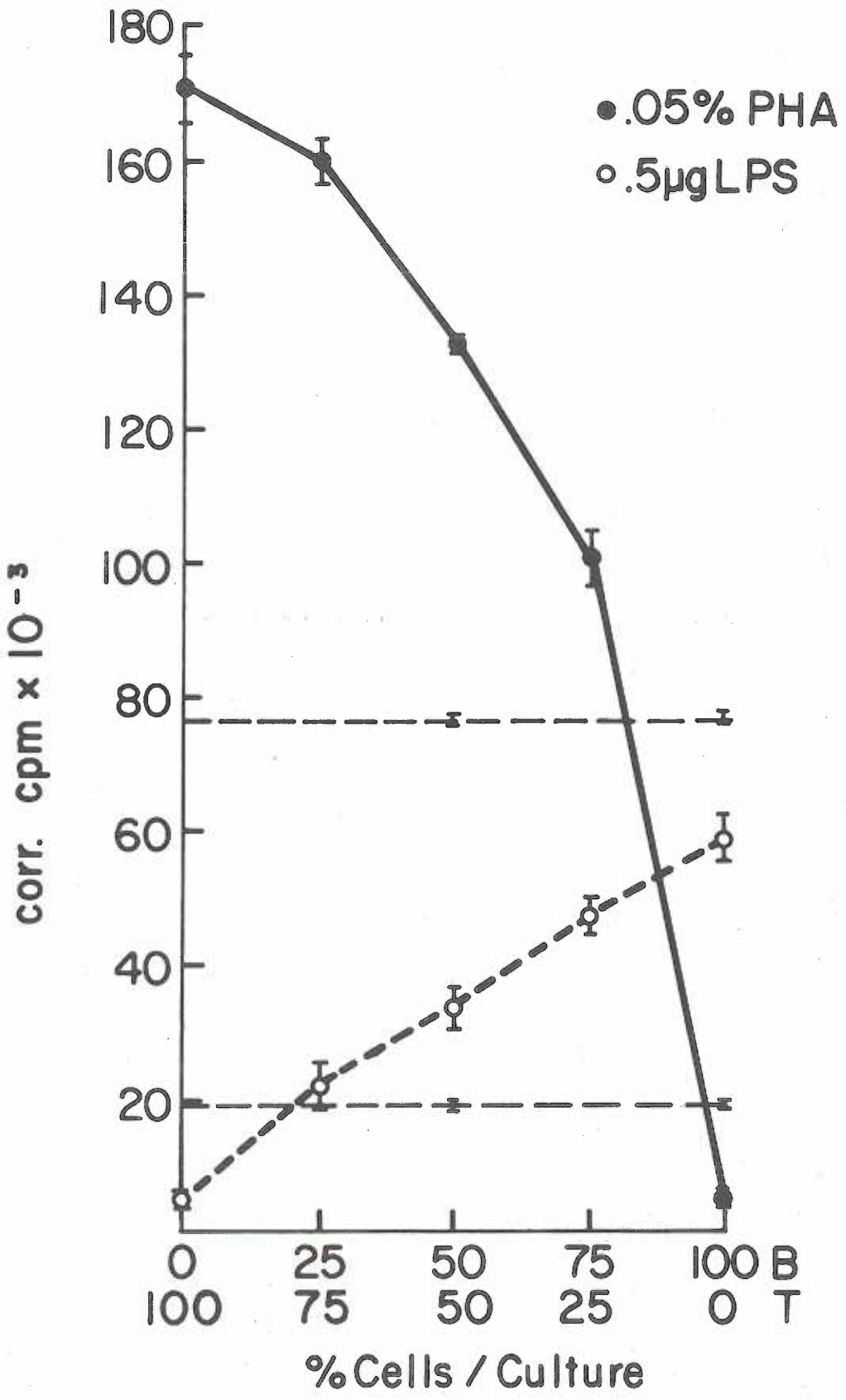

180
160
140
120
100
80
60
40
20
corr. cpm x 10⁻³
3
4.5
6
cells / culture x 10⁵
△ B cells + .05% PHA
● B cells + .5 µg LPS
○ T cells + .05% PHA
■ T cells + .5 µg LPS

Figure 17: Responses of B and T cell mixtures to mitogens. Various proportions of normal B and T cells, at a constant number of $6X10^5$ total cells per culture, were stimulated with 0.05% PHA (closed circles) or 0.5μg LPS (open circles) and harvested at 72 hours. Responses of unfractionated spleen cells to PHA and LPS are represented by the top and bottom dashed lines respectively.


180
160
140
120
100
80
60
40
20
corr. cpm x 10⁻³
● .05% PHA
○ .5µg LPS
0 25 50 75 100 B
100 75 50 25 0 T
% Cells / Culture

a trypan blue cytotoxicity assay with anti-theta serum and guinea pig complement. Representative data average from five experiments is presented in Table IV. It can be concluded that both B and T cell populations isolated from the monolayers were enriched at least two fold over the unfractionated population, and it appears that the B cell population was probably more pure than the T cell population. Results were similar when sheep anti-theta was used instead of rabbit anti-theta and when % remaining viable cells was calculated and compared with the cytotoxic index.

B and T Lymphocyte Responses to Antigen:

Several experiments were performed to assess the responses of B and T cells to antigen. Mixtures of various numbers of immune B and T cells were cultured with 5μg of WST, and the results from two experiments are shown in Figure 18. There was an optimum ratio of T to B cells which extended over a broad range. The best response was usually obtained with 60 to 75% T cells (25 to 40% B cells), and a response comparable to that of unfractionated spleen was usually obtained with 20 to 35% T cells (65 to 80% B cells). The response was not directly related to either the number of T cells or the number of B cells. However, it could not be concluded from this type of experiment whether both B and T cells were responding to direct interaction with antigen or whether nonspecific factors were contributing to the cooperative response.

In many experiments, including those in Figure 18, it was observed

Table IV: Anti-theta cytotoxicity of B and T cells. Killing of spleen cells, thymocytes, bone marrow cells, or purified B or T cell fractions was measured using a 1/2 dilution of rabbit anti-theta serum and guinea pig complement. Results represent the average of five experiments.

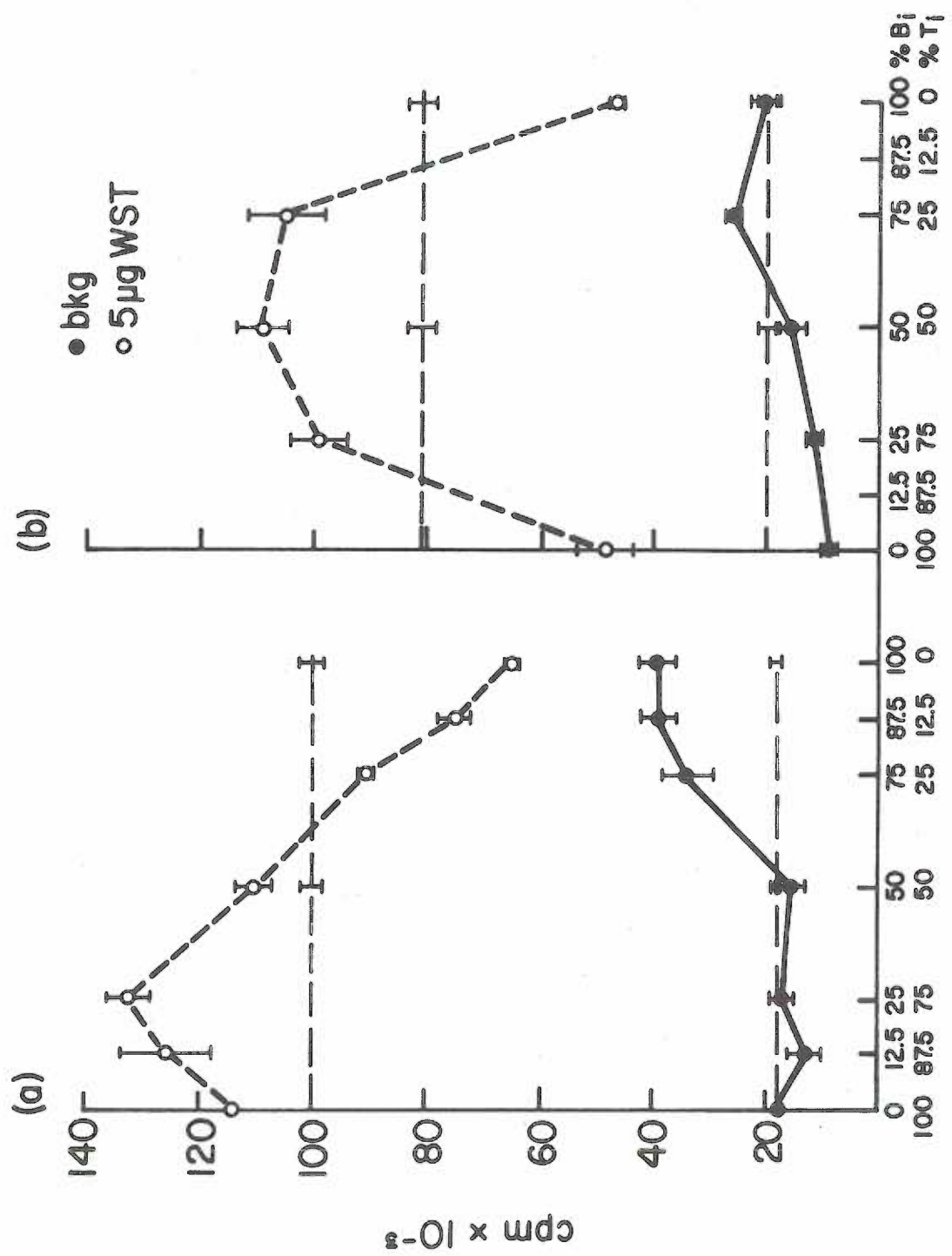
TABLE IV

Anti-Theta Cytotoxicity

antiserum	cells	C.I.[1]
RaBAθ (1/2)	spleen cells	38
	thymocytes	>98
	bone marrow cells	<5
	isolated T cells	86
	isolated B cells	7

1. cytotoxic index = $\frac{\%\ \text{dead E} - \%\ \text{dead C}}{100 - \%\ \text{dead C}} \times 100$

Figure 18: Responses of immune B and T cell mixtures to WST. Two experiments are shown (a and b) with spleen cells from LAF_1 mice immunized twice with 10μg of WST on alum. Various proportions of immune B (B_i) and immune T (T_i) cells, at a constant number of $6X10^5$ total cells per culture, were incubated for 120 hours in the presence or absence of 5μg of WST. Background cpm (closed circles) and responses to antigen (open circles) are plotted as average cpm/culture ± standard error. Backgrounds and responses to WST of unfractionated spleen cells are represented by the bottom and top dashed lines respectively.


(a)
(b)
● bkg
○ 5µg WST
cpm x 10⁻³
140
120
100
80
60
40
20
0 12.5 25 50 75 87.5 100
100 87.5 75 50 25 12.5 0
% Cells / Culture
% B_i
% T_i

that cell background was relatively constant until the immune B cells exceeded 50% of the total cell number, and it then increased with increasing numbers of B cells. This increase was not seen with increasing numbers of normal B cells or with immune cells obtained prior to day 5 after boosting. Furthermore, high background was associated with the glass adherent immune population isolated in a single experiment.

Responses of immune B or T cells, either alone or combined with normal cells to maintain a constant number of cells per culture, were measured to various dilutions of WST. The dose response curves for the isolated populations were similar and were indistinguishable from the dose response curve of unfractionated spleen (not shown). Therefore, all experiments were performed with an optimum dose of 1 to 10μg of WST per culture.

Table V shows the responses of immune B and T cells and unfractionated spleen cells to 5μg of WST. B and T cell populations responded to antigen when cultured alone, but responses were improved by the addition of normal cells. Responses of $3X10^5$ unfractionated immune cells alone or supplemented with unfractionated normal cells have been compared in other experiments, and the response is generally improved by 20 to 50% by the addition of normal filler cells. Although the magnitude of responses was generally highest when normal B cells were used for supplementation, there was little difference between normal B cells and unfractionated cells when stimulation indices were compared. Normal T cells appeared to provide the least help, possibly due to decreased numbers of macrophages. B cell responses

Table V: Role of normal cells in response to antigen. Unfractionated spleen cells (unf), B cells, or T cells ($3X10^5$/culture) from LAF_1 mice immunized twice with 10μg of WST on alum were cultured alone or with $3X10^5$ normal B, T, or unfractionated cells. Cultures were stimulated with 5μg of WST and harvested at 120 hours.

TABLE V

Role of Normal Cells in Response to WST

normal cell supplement	immune cells unfractionated	B	T
none	ND[1]	2,569 (1.4)[2]	38,864 (5.4)
B	72,692 (4.6)	5,434 (1.7)	100,501 (7.7)
T	33,176 (2.3)	3,107 (1.6)	50,510 (7.1)
unf	36,388 (3.3)	13,146 (2.9)	45,538 (5.2)

1. not done
2. cpm corrected for cell background (stimulation index)

in this experiment were very low because cells were obtained three days after boosting, an observation that will be commented on later.

Because the in vitro response to WST occurred during a restricted time period after boosting (see Figures 9 and 10), it was of interest to evaluate B and T cell responses during this time to determine if there are any changes in the relative responsiveness of B and T cells and whether there is any cooperation between B and T cells. Spleens were excised 2 to 8 days after boosting with 10μg of WST on alum; immune B and T cells were cultured alone, together, or with normal cells, and their responses were compared to the response of unfractionated spleen. Results of four experiments are presented in Figures 19 and 20, and data from these experiments is tabulated in Table VI to show the presence or absence of B cell-T cell cooperation.

When spleen cells were assayed three days after boosting (Figure 19a), the response to antigen appeared to be due primarily to T cells; the responses of T cells supplemented with normal cells were similar to that of unfractionated spleen, and the responses of mixtures of immune B and T cells were generally equivalent to the responses of immune T cells cultured with normal B cells. The exception to this last statement was the mixture containing 75% immune B cells and 25% immune T cells where the response was about 1.3 times greater than that expected from addition of B and T cell responses. This was the earliest indication of cooperation or synergism between B and T cells (see Table VI). At day three the B cells were responding, and their stimulation indices were significant because cell background was low. However, the magnitude of the B cell response at this time was at

Figure 19: B and T cell responses to WST - day 3 and day 4 after boosting. Spleen cells from LAF_1 mice immunized with 10μg of WST on alum were assayed three (a) or four (b) days after a secondary immunization. Various numbers of isolated immune B (B_i) or immune T (T_i) cells were cultured alone, together, or with normal cells (B_n or T_n) in the presence or absence of 5μg of WST; responses are presented as average cpm/culture ± standard error with stimulation indices indicated above each column. Responses were compared to the response of unfractionated immune cells (unf_i).

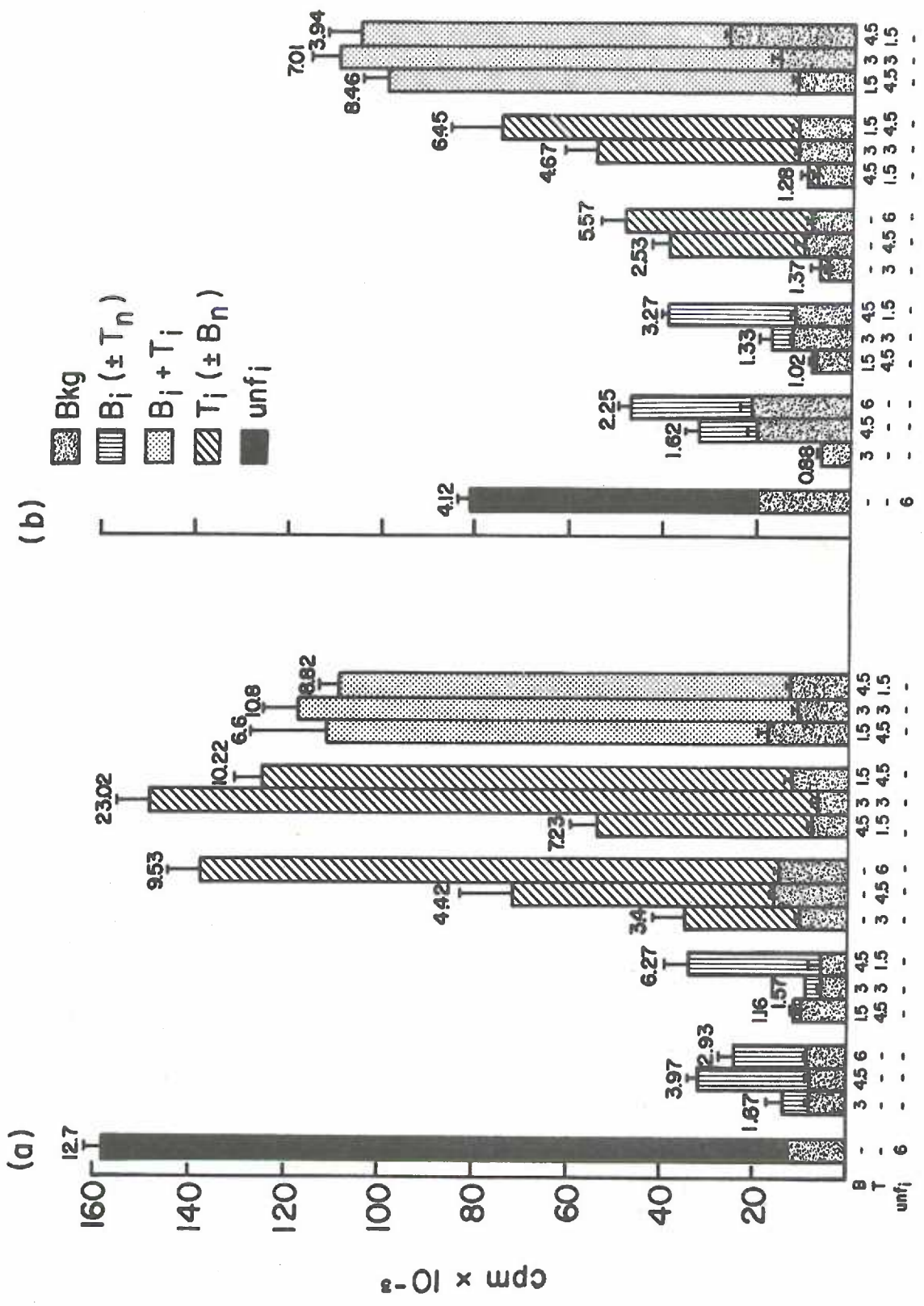

(a)
(b)
Bkg
B_i (± T_n)
B_i + T_i
T_i (± B_n)
unf$_i$
cpm x 10^{-3}
160
140
120
100
80
60
40
20
B
T
unf$_i$
12.7
1.67
3.97
2.93
1.16
1.57
6.27
34
4.42
9.53
23.02
7.23
10.22
6.6
10.8
8.82
4.12
0.88
2.25
1.62
1.02
1.33
3.27
1.37
2.53
5.57
1.28
4.67
6.45
8.46
7.01
3.94

Figure 20: B and T cell responses to WST - day 7 and day 8 after boosting. Spleen cells from LAF_1 mice immunized with 10μg of WST on alum were assayed seven (a) or eight (b) days after a secondary immunization. Various numbers of isolated immune B (B_i) or immune T (T_i) cells were cultured alone, together, or with normal cells (B_n or T_n) in the presence or absence of 5μg of WST; responses are presented as average cpm/culture ± standard error with stimulation indices indicated above each column. Responses were compared to the response of unfractionated immune cells (unf_i).

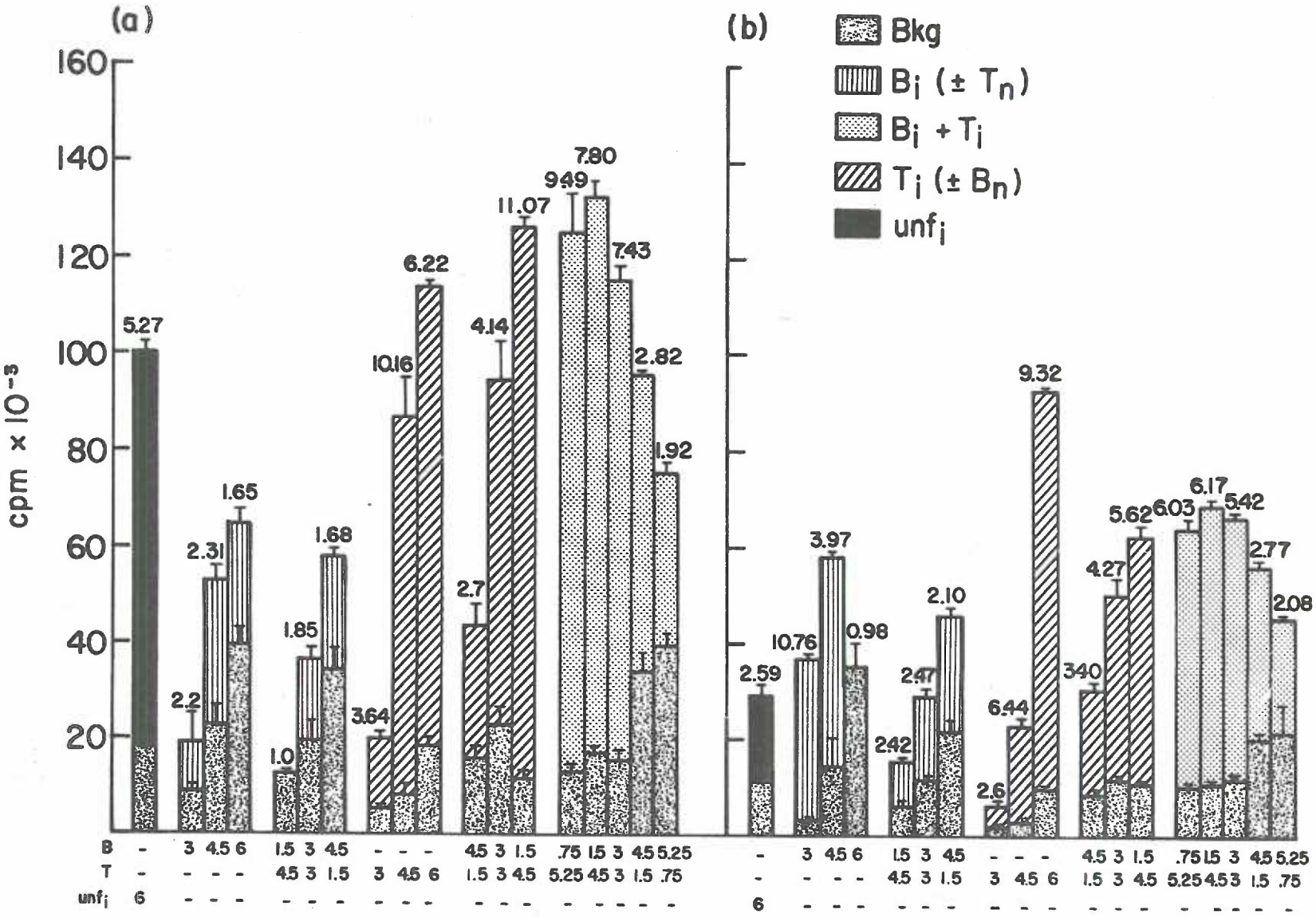

(a)
(b)
Bkg
B_i (± T_n)
B_i + T_i
T_i (± B_n)
unf_i
cpm × 10^{-3}
160
140
120
100
80
60
40
20
5.27
2.2
2.31
1.65
1.0
1.85
1.68
3.64
10.16
6.22
2.7
4.14
11.07
9.49
7.80
7.43
2.82
1.92
2.59
10.76
3.97
0.98
2.42
2.47
2.10
2.6
6.44
9.32
3.40
4.27
5.62
6.03
6.17
5.42
2.77
2.08
B - 3 4.5 6 1.5 3 4.5 - - - 4.5 3 1.5 .75 1.5 3 4.5 5.25 - 3 4.5 6 1.5 3 4.5 - - - 4.5 3 1.5 .75 1.5 3 4.5 5.25
T - - - - 4.5 3 1.5 3 4.5 6 1.5 3 4.5 5.25 4.5 3 1.5 .75 - - - - 4.5 3 1.5 3 4.5 6 1.5 3 4.5 5.25 4.5 3 1.5 .75
unf_i 6 - - - - - - - - - - - - - - - - - 6 - - - - - - - - - - - - - - - - -

Table VI: Synergism between immune B and T cells. Results from Figures 19 and 20 were corrected for cell background, and the responses of mixtures of immune B and T cells were compared to the expected additive response calculated from cultures of immune and normal cells.

TABLE VI

Synergism Between B and T Cells

Days after boosting	cells X 10^5 [1] per culture	observed [2] corr. cpm	expected [3] corr. cpm	% [4] synergism
3	$1.5B_i + 4.5T_i$	94,667	114,613	-17
	$3B_i + 3T_i$	106,761	145,540	-27
	$4.5B_i + 1.5T_i$	96,340	74,430	29
4	$1.5B_i + 4.5T_i$	87,733	63,381	38
	$3B_i + 3T_i$	93,756	47,206	99
	$4.5B_i + 1.5T_i$	78,232	29,139	168
7	$1.5B_i + 4.5T_i$	115,622	114,849	1
	$3B_i + 3T_i$	99,799	88,592	13
	$4.5B_i + 1.5T_i$	61,842	51,010	21
8	$1.5B_i + 4.5T_i$	57,819	60,820	-5
	$3B_i + 3T_i$	54,120	55,883	-3
	$4.5B_i + 1.5T_i$	55,016	45,811	20

1. B_i, immune B cells; T_i, immune T cells

2. response of $B_i + T_i$ mixtures to 5μg WST/culture

3. antigen response of $(B_i + T_n) + (T_i + B_n)$ where T_n and B_n are normal cells used for supplementation

4. $\%\ \text{synergism} = \left(\frac{\text{observed corrected cpm}}{\text{expected corrected cpm}} - 1\right) \times 100$

most 20% that of the T cell response.

When spleen cells were assayed at day 4 (Figure 19b), B and T cell responses to WST were more equal, and together their responses were greater than expected from addition of responses of immune cells cultured with normal cells. The amount of B cell-T cell synergy is presented in Table VI. Synergism increased from 38% with 25% B cells to 168% with 75% B cells; this suggests that the antigen specific response of B cells may be augmented by low numbers of responding immune T cells.

Results from experiments performed 7 and 8 days after boosting are shown in Figure 20. In both of these experiments B cell backgrounds were high, making it difficult to evaluate their responses. Responses of unfractionated cells, B cells, and T cells were still high at day 7 but had begun to decrease by day 8; this decrease was especially noticeable in the unfractionated population. Also at day 8, B and T cell responses exceeded the response of unfractionated spleen by up to three fold, and this was never seen early after boosting. In both experiments in Figure 20, responses of immune B and T cells cultured together were additive rather than synergistic (Table VI).

Several other observations can be made from these four experiments (Figures 19 and 20). Responses of both immune B and T cells were generally related to the number of cells per culture, and responses were better if cell densities were kept constant by the addition of normal cells. T cells responded better than B cells in terms of the maximum cpm and the minimum number of cells which would respond; this was especially evident if stimulation indices were compared.

The changes in the patterns of reactivity discussed above (Figures 19 and 20; Table VI) have been observed in repeat experiments. Very early after boosting (day 2 to 3) T cells make the major contribution to the response; slightly later (day 4 to 5), there is synergism between B and T cell responses; and later still (day 7 to 8), the responses are additive and exceed the response of unfractionated spleen. Responses of unfractionated cells are generally maximum at the time when synergism between B and T cells is evident and very low later in the response.

B and T cell responses to the purified antigens were also evaluated to determine the minumum number of responding cells. Table VII presents the results of two experiments which demonstrate that B and T cells can both respond to AgB and to AgA. Response patterns were similar to those observed with WST; T cells responded better than B cells, and responses were generally related to the number of immune cells per culture. Both T and B cells appeared to respond better to AgA than to AgB, even though in one of the experiments reported in Table VII the AgB and AgA responses of unfractionated spleen were very similar. This might suggest that optimum doses were not employed although there is no other evidence to support this hypothesis; dose response curves using the purified antigens were indistinguishable for the fractionated and unfractionated cells.

Since B lymphocytes responded to WST, AgA, and AgB, there must be multiple populations of responding B lymphocytes. By definition, the specificity of serum antibody is the same as the specificity of the surface receptors on the B lymphocytes secreting that antibody. Therefore, there must be at least two distinct populations

TABLE VII

B and T Cell Responses to AgA and AgB

days since boosting	cells X 10^5 per culture	background[1]	response to[2] 5μg AgA	response to 5μg AgB
3	$6unf_i$	12,489±1,414	68,476±3,882 (5.48)	73,916±1,416 (5.92)
	$1.5B_i + 4.5T_n$	9,853±1,259	9,486±820 (0.96)	9,201±385 (0.93)
	$3B_i + 3T_n$	5,461±520	28,206±620 (5.16)	10,653±567 (1.95)
	$4.5B_i + 1.5T_n$	5,387±727	29,549±4,523 (5.49)	16,970±283 (3.15)
	$1.5T_i + 4.5B_n$	7,396±797	61,762±5,597 (8.35)	18,015±5,358 (2.44)
	$3T_i + 3B_n$	6,468±839	83,460±2,719 (12.9)	33,157±7,214 (5.13)
	$4.5T_i + 1.5B_n$	12,264±1,338	88,471±5,583 (7.21)	35,020±3,049 (2.86)
8	$6unf_i$	11,205±1,371	43,097±4,633 (3.85)	23,986±2,584 (2.14)
	$1.5B_i + 4.5T_n$	6,472±1,015	30,219±1,499 (4.67)	10,405±485 (1.61)
	$3B_i + 3T_n$	22,517±2,080	54,570±4,126 (2.42)	16,364±247 (0.73)
	$4.5B_i + 4.5T_n$	21,957±2,238	50,656±6,387 (2.31)	38,074±2,344 (1.73)
	$1.5T_i + 4.5B_n$	9,022±850	38,773±4,430 (4.30)	21,152±2,562 (2.34)
	$3T_i + 3B_n$	11,786±1,065	69,282±4,438 (5.88)	25,426±3,367 (2.16)
	$4.5T_i + 1.5B_n$	11,169±864	88,437±3,133 (7.92)	43,802±5,144 (3.92)

1. background = cpm of cultures without antigen
2. cpm ± standard error (stimulation index)

of responding B cells, one responding to AgA and the other to AgB. If this is true, you might expect that responses to AgA and AgB in the same culture would be additive and that the response to WST might be greater than either the response to AgA or the response to AgB. I have attempted addition experiments with the purified antigens, and the results neither support nor refute the idea of two responding cell populations. In all the experiments I have performed with isolated cells, I have never seen a WST response significantly greater than the responses to purified antigens. However, the concentration of AgB in WST is much greater than the concentration of AgA, and the optimum stimulatory dose of WST may reflect only AgB stimulation; WST is toxic at doses of >50μg/culture, and it may take even greater quantities to obtain an effective concentration of AgA.

Although T cells also responded to all antigen fractions, it cannot be concluded how many carrier specific populations are involved. The data could be interpreted to mean that there is a single carrier determinant common to all antigen fractions.

In order to clarify the numbers of responding populations, and also to confirm the presence of a carrier determinant on AgD, AgD inhibition of the B and T cell responses to WST, AgA, and AgB was studied (Table VIII). AgD inhibited the B cell responses to WST and AgB but not to AgA, and this confirms that there are at least two populations of responding B cells. AgD inhibited the T cell responses to all antigens suggesting that there is probably a common carrier determinant. If there are two populations of responding T cells, then AgD would have to have two distinct carrier determinants as well as the hapten determinant; this seems unlikely because of its small size.

Table VIII: Antigen D inhibition of B and T cell responses to antigen. Unfractionated spleen cells, B cells or T cells from LAF_1 mice immunized twice with 10μg of WST on alum were excised 3 to 8 days after boosting and cultured with various amounts of AgD in the presence of 5μg of WST, AgA, or AgB. Maximum inhibition was calculated from 120 hour cultures, and the average results from two experiments are presented.

TABLE VIII

Antigen D Inhibition of B and T Cells

cells X 10^5 per culture	stimulating[1] antigen	maximum inhibition (%)
$6unf_i$	WST	91
	AgA	78
	AgB	80
$4.5B_i + 1.5T_n$	WST	88
	AgA	0
	AgB	100
$4.5T_i + 1.5B_n$	WST	91
	AgA	74
	AgB	91

1. all antigens at 5μg/culture
2. unf_i, unfractionated immune spleen cells; B_i, immune B cells; B_n, normal B cells; T_i, immune T cells; T_n, normal T cells

DISCUSSION

Antibody Responses to Timothy Pollen:

Several variables influenced homocytotropic antibody production in LAF_1 and C3H mice; these included antigen form, antigen dose, adjuvant, and mouse strain.

Both WST and AgB-Asc induced high titers of homocytotropic antibodies to timothy pollen antigens in LAF_1 mice. It is difficult to compare responses to WST and AgB-Asc directly since in equivalent antigen doses there is approximately two times more AgB in the crude extract, and the relationships between hapten and carrier determinants in the two preparations cannot be evaluated. Moreover, when WST is used for immunization, precipitating antibody is induced to the AgA determinant, and the effect of this on homocytotropic antibody production has not been investigated. In vitro studies suggested that there is a single carrier determinant, and if this is true, then IgG antibodies could suppress the secondary IgE response at the level of the T lymphocyte. Furthermore, different populations of B cells could be competing for the same carrier-specific cells or for nonspecific components of the immune response such as macrophages; these influences could modulate the relative amounts of homocytotropic and precipitating antibodies.

Similar interactions could also influence the response to AgB-Asc since B cells producing antibodies to Asc and AgB might be interacting with one population of Ascaris-specific helper cells. However, in

this system I cannot rule out the existence of helper cells specific for a carrier determinant of AgB which is accessible after conjugation. It is of interest that conjugated Ascaris induced three to four fold lower homocytotropic antibody titers than the native Ascaris, and yet no decrease in antigenicity could be demonstrated. Conjugation might alter carrier determinants or some type of antigenic competition could be operating.

A complete dose response study was not performed with either alum or CFA. Therefore, the effect of adjuvant cannot be evaluated. However, generalizations can be made about the relative immunogenicities of high and low antigen doses. Low doses of 1 or 10μg induced high IgE titers which could be boosted up to six times (Figure 3). Higher doses of 250μg resulted in low homocytotropic antibody titers which were absent after 2 or 3 immunizations. In general, IgG antibodies increased with increasing immunizing dose and IgE antibodies decreased (Table I). The IgG_1 to IgE ratio was greater with 10 than with 1μg doses, and although precipitating antibodies were demonstrable in all sera from WST immunized mice, they were maximum after high dose immunization.

These results confirm the work of others (e.g. Hamaoka, Newburger, Katz, and Benacerraf, 1974); IgE and IgG production could not be completely dissociated by changing antigen dose. However, in the timothy pollen system, IgE synthesis in the absence of IgG synthesis could be achieved by immunization with AgB-Asc, and different proportions of IgE and IgG could be obtained by immunization with different doses of WST. Furthermore, IgE and IgG antibodies were directed against

different determinants, whereas in the experiments of Ishizaka et al. and Katz and coworkers both were directed against DNP. Therefore, the timothy pollen system might provide a good model for looking at relative differences in the IgG and IgE systems.

The ability to produce high titers of reaginic antibodies is a genetic trait (Vaz and Levine, 1970), and therefore two strains of mice were examined for their abilities to make homocytotropic antibodies against timothy pollen. C3H mice had been shown to give high titers of homocytotropic antibodies to penicillin haptens (Levine and Vaz, 1970), and LAF_1 were reportedly good responders to ovalbumin. LAF_1 mice proved to be better responders to timothy pollen and were therefore utilized in all subsequent experiments.

The immunogenicity of AgD (Table II) is probably due to a low level of immunogenic material since a boosterable IgE response was obtained with up to 1mg immunizing doses; with WST, boosterable IgE responses are obtained with 10μg but not with 250μg doses. Antigen D immunogenicity could be explained in three ways. First, AgD in adjuvant might behave as a thymus-independent antigen and stimulate B cells directly. However, there is no published evidence for IgE synthesis to T-independent antigens. Second, although AgD can interact with T cells in vitro, it may be unable to trigger them in vivo, and adjuvant might facilitate proper presentation; AgD has not been tested for its ability to prime a helper cell population in vivo. Alternatively, adjuvant could facilitate insufficient B cell-T cell interactions. Since AgD did not induce formation of detectable amounts of precipitating antibodies, this supports the observations of Hamaoka, Katz,

and Benacerraf (1973) and Okudaira and Ishizaka (1975) which suggested a lower threshhold for IgE-B cell activation.

Finally, AgD immunogenicity could be the result of antigen contamination. Doses of less than 1μg of WST were not tested for immunogenicity, and therefore I do not know the minimum amount of antigen required for induction of an immune response. The following conflicting observations do not allow me to rule out antigen contamination. Contamination of AgD could not be detected by evaluating its ability to induce P-K reactions or histamine release in test situations where the minimum challenge dose of antigen could be titrated. On the other hand, AgD did induce PCA reactions in mice at doses of 250 to 500μg per animal. However, there are some data implying successful PCA challenge with monovalent material (Mancino, Tigelaar, and Ovary, 1969), so this does not prove the presence of antigen. Also, although AgD was not stimulatory in vitro, it was toxic at doses of more than 75 to 100μg per culture, and these doses may have still been too low to allow detection of a stimulatory antigen contaminant.

Because of the immunogenicity of AgD, it could not be used for in vivo suppression studies. I did try to suppress IgE production with doses of AgD which were only weakly immunogenic and was unable to demonstrate a decrease in homocytotropic antibody titers. In order to pursue in vivo suppression studies, AgD will have to be altered or administered in such a way that it does not induce antibody production.

Specificities of Antibodies to Timothy Pollen:

The specificities of the antibodies produced to WST were similar to those of human antibodies. That is, homocytotropic antibodies were directed against AgB and precipitating antibodies against AgA. Differences in the avidities of homocytotropic antibodies could not be detected by AgD inhibition studies. I had anticipated that antibodies obtained after multiple immunizations might be inhibited with lower doses of AgD, but this system was apparently not sensitive enough to detect differences if they did exist. Other investigators have detected differences in IgE avidities by comparing PCA challenge with different antigen doses (Katz, Hamaoka, and Benacerraf, 1973).

The most important discovery of these initial experiments was that three distinct groups of mice can be defined, and it was hoped that distinctions between them could also be seen in vitro. These groups are distinguished by the class, specificity, and amounts of antibodies produced, and predictions can be made about their lymphocyte populations. First, mice immunized with AgB-Asc produce only homocytotropic antibodies against timothy pollen and therefore have two populations of B cells; IgG_1 and IgE producing B cells are both specific for AgB. T cells in these mice might recognize a carrier portion of AgB as well as Ascaris. In order to establish carrier specificity conclusively, isolated T cells will have to be stimulated with AgB and Ascaris; as discussed later, indirect evidence indicates that AgB is probably acting as a carrier in these mice.

The second group of mice which can be defined produce homocyto-

tropic and precipitating antibodies after immunization with 10μg of WST on alum and must, therefore, have at least three populations of B cells, the two mentioned above plus IgG-B cells specific for AgA. They probably also have IgM-B cells and possibly IgA-B cells as well. These mice have at least one population of helper T cells specific for a carrier determinant of timothy pollen. Finally, mice immunized with 250μg of WST in CFA produce slight amounts of homocytotropic antibodies, but the major response after boosting is precipitating antibodies directed against AgA. Therefore, they have the same populations of B and T cells as the second group but in different proportions; most B cells must be IgG-B cells synthesizing antibody against AgA.

In Vitro Methods - Lymphocyte Transformation:

The in vitro lymphocyte transformation system was capable of detecting antigen-specific proliferation of immune LAF_1 spleen cells; normal cells or lymphocytes from mice primed with a different antigen did not respond in vitro to timothy pollen antigens (Figure 8). Some of the variables contributing to the transformation response were discussed in the Introduction; I investigated several others.

First, since I was interested in analyzing responses of different numbers of isolated B and T cells, it was necessary to study the importance of cell density. Others have shown that cell contact enhances proliferation (Peters,1974; Thorpe and Knight, 1974; Shearer and Parker, 1975), and this was confirmed. Optimum responses to antigen

were obtained with $6X10^5$ cells per culture, responses of lower numbers of cells were augmented by using normal cells as filler, and with greater cell numbers medium became exhausted. Therefore, isolated B and T cells were supplemented with normal cells in most experiments.

Second, two media supplements were evaluated. When isolating B and T cells the time involved is long, and bicarbonate-buffered RPMI becomes slightly alkaline; therefore, increasing the buffering capacity with HEPES would have been desirable. Also, in the manipulation of cell populations, I was concerned with the possible disruption of the balance between lymphocytes and adherent cells and therefore tested the effects of 2-mercaptoethanol which can enhance proliferation of non-adherent cells (Bevan, Epstein, and Cohn, 1974). Similar results were obtained with HEPES at 10 to 40mM or with 2-ME at $5X10^{-4}$ to 10^{-6}M. Background proliferation was increased two to three fold whereas antigen and mitogen stimulation were enhanced only slightly. Therefore, these reagents greatly decreased stimulation indices and were not used in subsequent experiments. Thorpe and Knight (1974) reported similar findings with HEPES.

The last variable which should be mentioned is the serum source. In all my experiments I used 5% fetal calf serum. Coutinho and Moller (1973) have postulated that FCS contains lymphocyte mitogens, and Shigi and Mishell (1975) detected bacterial contamination in some batches of FCS. Therefore, natural serum products or bacterial compoents such as LPS or proteolytic enzymes might stimulate mouse lymphocytes in cultures and account for the high backgrounds in my experiments. Other investigators report lower backgrounds with autologous or human

serum[4].

The variation in cell backgrouns with different spleen cell sources was significant. Background increases of up to 3 fold were seen 5 to 8 days following immunization (Figures 9 and 10). The cells responsible for the background were found in the adherent population and were also associated with the isolated B cell fraction. Osborne and Katz (1973) and Kirchner, Chused, Herberman, Holden, and Lavrin (1974) also attributed high background to adherent cells; the former group postulated that B cells were responding to macrophage-associated antigen, and the latter felt macrophages themselves were dividing in vitro.

The major problem with high backgrounds came in interpreting results obtained with greater than $3X10^5$ immune B cells per culture. If the background is due to lymphocytes responding to macrophage-associated antigen, then evaluating results calculated as corrected cpm or stimulation index would give low estimates for the size of the responding population. For this reason, I have reported cell backgrounds, uncorrected cpm for antigen stimulated cultures, and stimulation indices for most experiments.

In Vitro Methods - EA Monolayer Cell Separation:

I chose the monolayer technique for separation of B and T cells for two reasons: first, it allowed recovery of both cell types, and second, it was simple. This method is based on the belief that only

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4. B. Gibbins, personal communication

B cells have receptors for the Fc portion of immunoglobulin (Basten, Miller, Sprent, and Pye, 1972). A potential problem is that some T cells may bind to the monolayers. Several groups (Anderson and Grey, 1974; Gyongyossy, Arnaiz-Villena, Soteriades-Vlachos, and Playfair, 1975) have recently reported a small subpopulation of mouse T cells with Fc receptors; these are presumably activated thymocytes. Therefore, although T cell contamination of B cells is minimal in my experiments, it could represent a population enriched for timothy pollen-reactive cells. It is impossible to rule this out at this time although three observations make it unlikely. First, Fc receptor-bearing T cells are more dependent on a proper antigen-antibody ratio for complex binding and seem to have fewer receptors than B cells (Gyongyossy, et al., 1975). Therefore, under the saturating conditions used for B cell adsorbtion, B cell binding to the monolayers should be favored. Second, AgD inhibition profiles of the isolated B and T cell populations were distinct (Table VIII). Finally, B cells were contaminated with an estimated 8% T cells which is equivalent to $0.36X10^5$ T cells in a culture containing $4.5X10^5$ B cells. Yet, fewer than $3X10^5$ T cells rarely responded to antigen. Therefore, the magnitude of the B cell response cannot be due entirely to T cells although T cells may augment proliferation.

Another problem I encountered was an increased lability of B cells. After 24 hours in culture, unfractionated spleen cells and unbound T cells were 80 to 90% viable whereas the B cells were only 40 to 65% viable. I do not know whether this is due to the ammonium chloride treatment, the absence of some critical cell type, or some

other factor; also, filler cells could improve the viability of B cells. However, this observation must be kept in mind when comparing B and T cell responses to antigen; the apparent superior response of T cells might be explained by a greater number of total immune cells.

Cell recoveries from the monolayers were good. An average of 80% of the applied cells could be recovered, and of these 60% adhered to the monolayers and 40% did not; these values agree well with the proportions of B and T cells in the mouse spleen (Miller, Basten, Sprent, and Cheers, 1971).

It has been established that with mouse lymphocytes LPS stimulates primarily B cells and PHA primarily T cells (Doenhoff, Janossy, Greaves, Gomer, and Snajdr, 1974). The purities of the two monolayer fractions as evaluated by mitogen stimulation were acceptable (Figure 16); T cells were 87% pure and B cells were 92% pure. Results with anti-theta and complement confirmed enrichment of the two populations (Table IV). I feel these degrees of enrichment are adequate to allow conclusions to be drawn about B and T cell responses if graded numbers of cells are cultured to allow determination of the minimum number of immune B or T cells necessary for response to antigen.

In Vitro Response to Antigen:

WST-immune spleen cells responded to all pollen fractions in vitro (Figure 8). With human peripheral blood lymphocytes, dose response curves are very sharp (Malley, Wilson, Barnett, and Perlman, 1971); in

the mouse they were extremely broad. The broad curves in the mouse could be partially the result of multiple populations of responding cells. In humans most antibody to timothy pollen is reaginic whereas in mice considerable precipitating antibody is made as well; furthermore, precipitating and reaginic antibodies are directed against different determinants and there are two classes of homocytotropic antibodies in the mouse. Different proportions of B and T lymphocytes may also contribute to the breadth of the response curves. Human peripheral lymphocytes are about 80% T cells, and since there appears to be only a single carrier determinant, heterogeneity of the response should be less than with mouse spleen which is about 60% B cells. Heterogeneity of receptor avidities, cell interactions, and the presence of different antigenic determinants in different concentrations in the antigen preparations might also contribute to the dose response profiles. However, both AgA and AgB were relatively homogeneous by immunoelectrofocusing, and any heterogeneity would thus have to be intramolecular.

No differences in optimum stimulatory dose could be detected when WST-immune spleen cells were assayed at different times after primary or secondary immunizations. Also, the magnitude of the response did not seem to be related to antibody titer. There could be several explanations for this. First, lymphocyte transformation may not be sensitive enough to detect differences in receptor avidities. Second, cell mediated immunity was not measured in these mice and might contribute significantly to the overall immune state. Third, homocytotropic antibody was the only class which was titered and used for direct comparison to lymphocyte transformation, but absolute amounts of homo-

cytotropic antibodies should be low compared to precipitating antibodies. Moreover, Tada and Ishizaka (1970) found most IgE plasma cells in the respiratory mucosa of humans and monkeys with very few in the spleen. The IgE-B cells may, therefore, be contributing very little to the magnitude of the response in my system.

Comparative studies with mice immunized in different ways gave disappointing results; differences between the groups could not be defined (Figures 14 and 15). I had expected mice immunized with WST to respond in vitro to WST, AgB, and AgA. I did not expect cells from AgB-Asc immunized mice to also respond to all antigens, but they did. This probably indicates that there are timothy specific helper cells in these animals which respond in culture to the carrier determinant on AgA which must be identical to or cross reactive with the carrier determinant on AgB. Since dose response curves with AgA were indistinguishable with different spleen cell sources, and since the AgA response of B cells from WST-immune mice could not be inhibited by AgD, I do not think that small amounts of AgB in the AgA preparation were responsible for stimulation. B and T cell responses of AgB-Asc-immune spleen cells to purified antigens will have to be evaluated.

Both B and T cells from mice immunized with WST responded to all antigen fractions (Figures 19 and 20), and a major question is whether B cells responded directly to antigen stimulation or as a result of T cell or macrophage help, either specific or nonspecific. Two pieces of evidence argue against a nonspecific T cell factor. First, cultures of immune T and immune B cells gave much greater responses than cultures of immune T cells and normal B cells. This suggests

that if T cells are elaborating a nonspecific factor, it preferentially enhances the response of immune B cells in the presence of antigen. The effects of T cell supernatants, generated with WST conjugated to Sepharose so that antigen can be removed, will have to be evaluated to determine whether there is a soluble blastogenic or potentiating factor. I have tested supernatants which contain antigen, and their stimulatory effects can be explained by the presence of antigen alone; furthermore, when assayed on normal cells, they had no apparent enhancing effects.

The second piece of data arguing against nonspecific T cell mitogenic factors is that it made little difference whether immune T cells were cultured with normal B cells, T cells, or unfractionated spleen cells. This indicates that if a nonspecific factor does exist, it either acts on all cell types or makes a minor contribution to the overall response. In some experiments, it did appear that normal B cells were better supplements than normal T cells, but this could be due to greater numbers of macrophages in the B cell population.

The results of experiments with isolated lymphocytes suggest that both B and T cells respond specifically to antigen in vitro. However, B cell responses might require small numbers of T cells. One experiment indicates this may not be the case. In the AgD inhibition studies, responses to high doses of AgA could be obtained even in the presence of concentrations of AgD which could completely inhibited responses to other antigens. If all T cells recognize the same determinant, and if AgD can inhibit their response, these observations suggest that B cells can divide in the absence of proliferating

T cells (Figure 12). Although the same results were obtained with isolated B and T cell populations, these experiments must be interpreted with caution since they could also be detecting two T cell or two B cell populations.

Antigen D Inhibition:

The fact that AgD was not stimulatory suggested it was monovalent for all determinants. It was thus used as a probe to further evaluate the number of discrete cell populations responding to timothy pollen in vitro.

One of the major problems with the AgD studies was the nonspecific toxicity of AgD. Of six preparations tested, five inhibited mitogen responses and responses of SRBC-immune spleen cells to SRBC. The sixth preparation demonstrated specific inhibition at doses of less than 100μg per culture and was used for all experiments reported. The toxicity of AgD is not due to the pigment since quercitin alone was not toxic. It could be related to the carbohydrate, cellobiose, although this has not been tested.

Antigen D inhibition was effective if AgD was present throughout the entire culture preiod; a three hour preincubation did not result in inhibition whereas it was effective with human peripheral lymphocytes. Again, this discrepancy between the two systems could be due to differences in the specificities and proportions of T and B cells and/or receptor avidities.

The results of the AgD inhibition studies (Figure 12, Table VIII)

confirmed that there are at least two populations of responding B cells defined by antigen specificity. One B cell population is inhibited by AgD and is presumably specific for AgB, and the other is probably reacting to AgA. AgD was also able to inhibit T cell responses. However, it cannot be concluded whether a single population of T cells is collaborating with IgE and IgG-B cells, whether two T cell populations with the same receptor specificities are involved, or whether there are two distinct populations inhibited by unique carrier determinants on AgD. The last possibility seems unlikely because of the small size of AgD (average molecular weight 5,000).

Since AgD could inhibit T and B cell responses, it must have both hapten and carrier determinants which by definition must be distinct. Physicochemical characteristics of AgD have been analyzed, and predictions can be made about the nature of the hapten and carrier determinants. Structurally, AgD consists of the quercitin pigment linked to cellobiose which is then attached to the polypeptide chain. The fragment D-3, which inhibits reaginic antibody and thus has the hapten, is composed of quercitin, cellobiose, and a single amino acid, tyrosine. Therefore, the antigenic determinant probably involves either the pigment or the sugar. Although cellobiose can be immunogenic (Gleich and Allen, 1965), it does not inhibit histamine release with anti-timothy reagin, and Mullan (1974) found no antigenic material in the carbohydrate fraction of timothy pollen isolated by hot phenol extraction.

In order to test antigenicity of the pigment, quercitin has been conjugated to a peptide, glutathione. The conjugate inhibits P-K

reactions and histamine release. Therefore, quercitin may be the antigenic determinant[5]. Results of in vitro lymphocyte transformation studies do not conflict with this conclusion. The quercitin-glutathione conjugate has been tested in vitro in three experiments, both with unfractionated spleen cells and isolated B cells. Quercitin-glutathione did not inhibit stimulation by WST but induced proliferation in a dose dependent fashion (maximum stimulation index = 3.6). This triggering of B cells intimates its role as the hapten determinant. The glutathione has a high charge density which may explain why the conjugate was able to stimulate in monovalent form whereas AgD and D-3 could not. Spengler, de Weck, and Geczy (1974) also induced proliferation of human lymphocytes to penicillin haptens in vitro.

From the experiments I have reported, the nature of the carrier determinant cannot be identified. However, it might be predicted that the polypeptide region is involved since it is the portion which D-3 lacks. Studies to evaluate this are currently in progress; β-glucosidase can cleave the polypeptide region from AgD and the quercitin-cellobiose and protein fractions can be evaluated. The lymphocyte transformation system, utilizing isolated B and T lymphocytes, will allow analysis of these fractions.

Differentiation of Responsive Cells:

The time course of spleen cell reactivity after immunization demonstrated kinetics of primary and secondary responses (Figures

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5. A. Malley, unpublished observation

9 and 10). Responses were maximum at day 6 to 10 following primary immunization and at day 5 to 6 after a boost. The ability to be stimulated in vitro then fell rapidly. There are at least three possible explanations for this. First, serum antibody titers rose slightly later than lymphocyte transformation responses and were maintained longer; feedback by antibody, alone or complexed to antigen, could suppress lymphocyte reactivity. Sell and Ansari (1975) recently presented data supporting this hypothesis; immune complexes from serum collected late after immunization when lymphocyte transformation had declined could inhibit the in vitro response of day 5 spleen cells. The fact that antibody titers were low after primary immunization with WST argues against this being the only mechanism involved. Second, suppressor activity could be generated late in the response. This is supported by the observation that B and T cells isolated from day 8 spleens responded better than unfractionated cells (Figure 20); suppressor cells appear to die off first in culture (Taussig, 1974b), and this selective death could be facilitated by the EA monolayer separation technique or the conditions for culture of the purified fractions. I was unable to show an inhibitory effect of day 8 spleen cells on day 4 spleen cells, but responses of late spleen cells could be enhanced by addition of normal cells suggesting that suppressor activity can be diluted out. The third possible explanation for the decline in lymphocyte transformation responses is that specific memory cells migrate out of the spleen. Other lymphoid tissue would have to be examined to rule this out.

Responses to B and T cell mitogens decreased after immunization,

and this could be the result of suppressive factors, suppressor cells, or changes in the spleen cell populations. The time course of unresponsiveness in my system correlated with the appearance of nonspecific suppressive factors in serum from animals with cell mediated immunity (Burger, Lilley, Reid, Irish, and Vetto, 1973). Decreased T mitogen responses in tumor-bearing mice occur after the peak cytotoxic response and have been attributed to suppressor macrophages or B lymphocytes (discussed by Kirchner, Chused, Herberman, Holden, and Lavrin, 1974). In my experiments anergy was not as great in the isolated B and T cell populations, and since both B cells and macrophages were primarily in the bound population, this could be explained by the loss of suppressor cells from the T cell population. Alternatively, the additional RPMI wash of fractionated cells might have diluted out a suppressive factor. It should be noted that whatever is suppressive to mitogen responses at day 5 could be responsible for suppression of antigen responsiveness later; cells in different states of differentiation could have different susceptibilities to its effects.

The changes observed in isolated and recombined B and T cell populations with time after boosting give additional insight into the differentiation of lymphocyte subpopulations (Figures 19 and 20; Table VI). In general, it appeared that antigen first activated T cells. B cells became responsive slightly later, probably as a result of interaction with T cells and macrophages, and their in vitro response could be amplified by T cells at the time of optimum response of unfractionated cells. Following this, T cell amplification and suppressive effects appeared to compete; in isolated populations they seemed

to be approximately equal, since B and T cell responses were additive, whereas in unfractionated spleen the suppressive effect predominated. Suppressive and amplifying effects could be due to cells or soluble factors and could be specific or nonspecific. At this time I have no data to indicate the mechanism of suppression.

My data is in agreement with several recent reports discussed in the Introduction. In Osborne and Katz's experiments (1973), B and T cell responses appeared to be additive, whereas in the experiments of Mugraby, Gery, and Sulitzeanu (1974) and Snippe and van Eyk (1974) there was cooperation and amplification between B and T cells. In all cases, lymphocytes were removed at variable times after immunization which could explain the differences seen. The possibility of there being sufficient numbers of T cells in the B cell populations to provide an essential accessory role has not been ruled out in any experiments. However, the fact that different cell separation techniques, including antibody plus complement treatment, passage over anti-Ig columns, and EA monolayer separation, have all resulted in the conclusion that T and B cells can both respond to antigen in culture, makes it unlikely that T cells are required for the B cell response to antigen.

SUMMARY AND CONCLUSIONS

Results of in vitro stimulation with purified pollen antigens and AgD inhibition experiments established the presence of multiple populations of antigen-reactive lymphocytes in spleens of timothy pollen immune LAF_1 mice. Cell separation studies further demonstrated that there are at least two populations of B cells, which apparently respond directly to either AgA or to AgB; moreover, the AgB reactive population might contain IgE and IgG_1 producing cells, the AgA population probably contains IgM and IgG producing cells, and there must be other minor populations which cannot be defined. In contrast, there appears to be only a single population of carrier reactive T cells since responses to all pollen fractions could be inhibited by AgD. Whether the same helper cells interact with IgE and IgG-B cells could not be established.

These studies also confirmed that AgD has a carrier determinant as well as a hapten determinant. Therefore, it is capable of interacting with both B and T cells and could suppress either cell type in vivo. Since T cells are more easily tolerized and remain tolerant for longer times, and since AgD suppression in humans is long lasting, the AgD effect is probably on the helper cell population. However, it cannot be ruled out that the suppressive effect is due to activation of a suppressor cell.

In humans who have been treated with AgD, the reagin titer is decreased but the blocking antibody titer against AgA is not. If there is a single population of helper cells, this might support the

contention that IgE-B cells are more sensitive to T cell regulatory influences than IgG-B cells. On the other hand, it could also support direct B cell inactivation or generation of antigen specific suppressor cells.

Hopefully this in vitro system can be used to more carefully define the site of AgD suppression. An AgD fraction must be obtained which is not immunogenic in mice. Two approaches might be taken. First, further fractionation or chemical modification might result in a fragment which still has a carrier determinant as defined by its ability to inhibit antigen-induced T lymphocyte transformation and proliferation; this could be used to induce T cell tolerance. Second, AgD or D-3 could be covalently coupled to a nonimmunogenic carrier, such as an isologous protein or D-amino acid copolymer, and used to suppress B cells directly. These fractions would have to be tested in vivo for their ability to suppress primary or secondary responses to WST or AgB-Asc. Spleen cells and isolated B and T cells from suppressed animals could then be evaluated in vitro to determine the cellular site of suppression. Screening of similar substances in rhesus monkeys might ultimately result in a compound which could be used successfully to treat humans with timothy pollen allergy.

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