1978

Effects of Horse Anti-Rabbit Thymocyte Serum and Adult Thymectomy on the Anti-Hapten Responses in Vivo.

Yee Hon Chin
Louisiana State University and Agricultural & Mechanical College

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EFFECTS OF HORSE ANTI-RABBIT THYMOCYTE 
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ANTI-HAPTON RESPONSES IN VIVO

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in
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by

Yee Hon Chin
B.S., Louisiana State University, 1974 
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ABSTRACT

The effect of horse anti-rabbit thymocyte gamma globulin (HATGG) treatment and short term adult thymectomy on the humoral antibody response of rabbits to the hapten, dinitrophenol was investigated. Rabbits treated with 30 mg HATGG on the same day or seven days after primary immunization with suboptimal doses of DNP-OVA (3 mg) showed enhanced anti-DNP responses which approached those seen in rabbits immunized with optimal doses of dinitrophenylated-ovalbumin, DNP-OVA, (10 mg). Rabbits thymectomized 30 days before they received 3 mg DNP-OVA produced primary anti-DNP responses identical to those seen in non-thymectomized animals immunized with 10 mg DNP-OVA, whereas rabbits thymectomized 7, 14, or 21 days before they received suboptimal dose of DNP-OVA produced low levels of anti-DNP antibody. In an attempt to characterize the effect GATGG and adult thymectomy exerted on the regulation of the immune response the antigenic competition model was used. Dinitrophenylated-bovine gamma globulin DNP-BGG, produced a hapten-specific secondary response when primed with 3 mg DNP-OVA for 3 days, if these animals were injected with 50 ug unconjugated BGG 7 days after the first antigen (DNP-OVA) injection. Rabbits injected with both keyhole limpet hemocyanin (KLH) and BGG seven days after the primary DNP-OVA injection showed suppressed anti-DNP responses after a secondary injection of DNP-BGG. When 30 mg of GATGG was injected at the same time both KLH and BGG
were administered or if the rabbit was thymectomized 30 days before the first antigen injection, the anti-DNP response after secondary DNP-BGG injection was restored to the levels seen in carrier-primed animals. Neither normal horse gamma globulin (NHGG), nor ATGG absorbed with rabbit-Ig did not alter the regulatory effect of ATGG. These results suggested that ATGG and adult thymectomy interfered with a regulatory function of T cells in the rabbits.

In the second aspect of this study it was shown that a soluble thymus extract prepared from the thymus' of either carrier-primed or normal donors suppressed the anti-DNP primary responses in recipients immunized with 10 mg DNP-OVA for 3 days. The regulatory activity was absent in extracts prepared from thymuses of cortisone treated donors and from a spleen extract prepared from thymectomized rabbit donors. The suppressive activity could be removed from the thymus extract by adsorption with ATGG-Sepharose 4B, but remained in the extract after adsorption with carrier, OVA-Sepharose 4B, NHGG-4B and Sepharose 4B alone. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the eluants from the ATGG-Sepharose 4B column by 3 M NaSCN revealed a characteristic band with a molecular weight of approximately 12000 daltons.
REVIEW OF LITERATURE

The discovery by Clamen et al (1961) and Miller and Mitchell (1969) that two cell types (helper and effector) participate in effecting cell-mediated and humoral immune responses has greatly increased our understanding of the complex immune mechanism. From X-irradiation and reconstitution studies, it was found that the thymus gives rise to thymus-derived cells or T cells and the mammalian equivalent of the avian bursa of Fabricius gives rise to precursors of antibody-producing cells or B cells (Pierce and Benacerraf, 1976). In the past few years, the importance of the macrophage as an accessory cell has also been established (Rosenthal and Shevach, 1975). Basically, a small B lymphocyte is believed to recognize antigen using a specific immunoglobulin surface receptor and, with the help of T cells, is triggered into clonal proliferation, followed by differentiation into plasma-cell-like antibody forming cells (Warner, 1974). The third cell type, the macrophage, is believed to be required in T cell proliferation and mediator production, i.e. the minimal antigenic recognition unit consists of both a macrophage of unspecified immunologic background and an antigen-specific T lymphocyte (Rosenthal and Rosenstreich, 1974).

During the process of characterization of T and B cell responses to several antigens and determining the cellular requirements for humoral and cell-mediated immune responses, it has become apparent that subpopulations with distinct "helper" and "suppressor" properties exist in the thymus-derived cell compartment. Considerable
evidence has accumulated that supports the concept that helper and suppressor T cells are distinct subpopulations of T cells.

The review that follows presents experimental evidence which supports the existence of a regulatory (suppressor) role for the T-cell and also examines the immunochemical and physiological characterization of these cells, which sets apart from helper (inducer) cells.

Subpopulations of T cells. In the mouse system, Raff and Cantor (1971) proposed a scheme to identify subpopulation of T cells, by utilizing life span, migration patterns and membrane antigens (alloantigen) as markers. One population of T cells, designated T-1 cells, are relatively immature, cortisone sensitive, short-lived, non-recirculating cells that possess abundant membrane theta antigen and migrate preferentially to the spleen. The T cells in the second subpopulation, T-2 cells, are more mature, long-lived cells, show less surface theta antigen, migrate preferentially to the lymph nodes, and are abundant in the recirculating pool of lymphocytes in peripheral blood and thoracic duct lymph. Animals treated with antilymphocyte serus (ALS) showed depleted T-2 cell levels, but showed no radical changes in T-2 cell numbers after adult thymectomy (Kappler et al., 1974; Cantor and Weissman, 1975). Helper T cells which cooperate during antibody synthesis to T cell-dependent antigens and amplifier cells which participate during cell-mediated immune responses appear to be T-2 cells and bear Ly 2,3 alloantigens on their membranes prior to antigenic stimulation. Furthermore, T-2 cells appear to account for most of the proliferative response to alloantigens in the mixed
lymphocyte reaction. There exists some evidence that Ly 2+, 3+ T-2 cells and T-1 cells bearing Ly 1+ alloantigen may regulate the magnitude and tempo of the immune responses (Cantor and Boyse, 1977).

Recent studies by Tada (1977) suggest the Ly 2+, 3+ T cells after antigen stimulation act on Ly 1+, 2+, 3+ T cells to induce non-specific suppression by the latter. Thus two populations of T cells could jointly mount T cell-mediated suppression, producing both long- and short term suppression in different models.

It is also possible that suppressor T cells may be relatively immature cells that could, under appropriate conditions, be responsible for helper T cell effects at a later stage in their life cycle after further differentiation. This differentiation may involve the switching of surface alloantigens (Bullock et al, 1978).

**Rabbit Thymocyte Membrane Surface Antigen.** T cell markers, as discussed in previous sections, have been identified in both the mouse and rat. The most widely studied thymocyte antigen is the Thy-1 (9) antigen found on mouse and rat thymocytes and also brain tissue. The presence of unique rabbit T cell antigens has been less studied and as a result are poorly defined. Studies by Fradelize et al (1973) and Shek et al (1976) described a thymus-specific antigen, which they termed "rabbit thymus lymphocyte antigen" (RTLA). RTLA was found to be present on more than 90% of the thymus cells, 50% of spleen cells, 60-70% of lymph node cells and on 12% of the circulating peripheral lymphocytes. RTLA at this time is not considered to be analogous to the theta molecule since RTLA antibody cannot be removed.
by absorption with rabbit brain. Redelman et al (1976) also reported a unique T cell antigen present on thymocytes isolated from nylon wool nonadhering cells in the spleen cell population. The lymphocytes that bear this antigen are susceptible to lysis with anti-thymocyte serum and complement, and thus presumably they could have thymocytes as their origin. Recently, Navran and Archer (1978) prepared unique T lymphocyte-specific surface antigen by 3M KCl extraction of rabbit thymocyte plasma membrane. The crude membrane extract was fractionated by gel electrophoresis. To identify which fraction contained the T cell antigen, anti-thymocyte serum cytotoxicity inhibition assays were done. By this method, the purified T cell antigen was found to have a molecular weight of approximately 12,000 daltons and contained approximately 2.5% carbohydrate. The authors suggested that the rabbit T cell antigen may exist in multiple forms since two gel fractions of the thymocyte membrane extract were able to inhibit the cytotoxicity of anti-thymocyte serum. Bash et al (1974) in earlier studies also suggested that the thymocyte antigen existed in multiple forms in rabbit T cell antigen. However, their data was not conclusive and the possibility existed that proteolytic digestion during the extraction procedure changed the form of the antigen.

In a brief note, DeLaNoue et al (1972) used an anti-serum produced in the horse against rabbit thymocytes to demonstrate the presence of a rabbit thymocyte specific antigen and an antigen common to all rabbit lymphocytes. These investigators reported that T cells were not present in the rabbit spleen, appendix, sacculus rotundus,
Peyer's patches on bone marrow. However, their findings have been widely disputed and in fact the rabbit appendix is indeed inhabited by T cells as shown by Ozer and Waksman (1977).

**Antigen-specific and non-antigen-specific suppression**

mediated by T cells. Non-antigen specific suppression which is mediated by T cells or by soluble factors elaborated by them played a significant role in (a) antigenic competition, (b) mitogen-induced suppression, and (c) in progressive infectious disease (malaria, trypanosomiasis, leprosy). Antigen-specific suppression mediated by T cells or their products appear to regulate certain immune responses such as reaginic (IgE) antibody production, and genetically restricted immune responses to some synthetic polymers and in the induction of tolerance.

First, nonantigen-specific suppression using antigenic competition as a model will be addressed, followed by a discussion of antigen-specific suppression, which Tada, through his elegant experiments, has proposed a molecular mechanism for this type of suppression in the mouse.

**Antigenic Competition.** Antigenic Competition occurs when the induction of an in vivo immune response to one antigen non-specificly suppresses the response to an unrelated antigen, one to seven days later (Liacopoulos and Ben-Efraim, 1975; Gershon, 1974; Taussig, 1973; Pross and Eidinger, 1974). A number of different
theoretical explanation have been offered to explain the immunological events which occur during antigenic competition. Some short-lived explanations are (a) competition for nutrients or biological space in lymphoid tissue, (b) interferences (competition) for the phagocytic and antigen presentation function of the macrophages (Reticulo-endothelial system blockade), and (c) competition among different haptenic determinants on the same carrier, all have been considered as possible mechanisms for antigenic competition (Taussig, 1973; Pross and Eidinger, 1974; Liacoupoulos and Ben-Efraim, 1975). Current information derived from systematically studied systems have strongly implicated the radio-resistant suppressor T cells and/or their soluble products as being responsible for regulating the depressed immune response to the second antigen.

It is generally accepted that antigenic competition can be evoked when: (1) the two competing antigens (priming and test) are injected into the same site (Eidinger et al., 1971), (2) the priming antigen precedes the test antigen by one to seven days (Brody and Siskind, 1972; Moller and Sjoberg, 1970) and (3) the priming antigen is given in greater than optimum concentration than the test (suppressed) antigen (Brody and Siskind, 1969; Duker and Dietrich, 1970). In addition, antigenic competition can be readily induced with T cell-dependent antigens, whereas T cell-independent antigens generally do not. However, triggered suppressor T cells which mediate antigenic competition will suppress antibody response to the inducing antigen and to unrelated T cell-dependent and T cell-independent
antigens. Furthermore, it has been shown by several groups of investigators that "nonspecific" stimulation of T cells with phytomitogens (lectins such as concanavalin A) can also produce antigenic competition-like effects (Sjoberg et al., 1973; Dutton, 1975a, b). Further, DNA synthetic responses to the T cell mitogen phytohemagglutinin in spleen of mice (Gershon et al., 1974a) and cultures of rat spleen and lymph node cells (Bash and Waksman, 1975) are suppressed if optimum doses of unrelated antigens had been injected into the test animal prior to harvest of the spleen cell. The cells that appear to regulate (inhibit) DNA synthesis in mitogen stimulated lymphocytes adhere weakly to glass wool, but are distinct from macrophages and the authors concluded that these antigenic competition-like phenomena are mediated by weakly adherent suppressor T cells.

Radovich and Talmadge (1967) were the first to suggest that a soluble suppressor factor, (secreted by a radio-sensitive T cells), may be responsible for the mechanism by which antigenic competition is induced. It has been reported that normal spleen cells when infused into animals manifesting antigenic competition, are unable to produce an immune response (Press and Eidinger, 1974; Gershon, 1974; Liacoupoulos and Ben-Efraim, 1975). However, when lymphoid cells harvested from the spleen of an animal manifesting antigenic competition are tested for their ability to respond outside of the host (in vitro culture), these cells develop normal responses to the same antigens that they fail to respond to in the suppressed animal. But antigenic competition was again observed if the competing antigen was
added to the culture. Dwyer and Cantor (1973) reported that lymphocytes harvested from anergic guinea pigs will respond in vitro to the same antigen to which the animal is unresponsive in vivo, and these lymphocytes can mediate delayed skin test responsiveness to the test antigen when transferred to normal recipients. Similar observations have been reported in studies of immunological tolerance (Liacopoulos and Ben-Efraim, 1975). The elaboration of soluble mediators into the micro-environment of the spleen and other lymphoid tissues by radio-resistant suppressor T cells, in the presence of antigen may influence the subsequent reactions to other antigens during the course of antigenic competition and related phenomena in the intact animals. Unfortunately, these soluble mediators have not been identified as yet.

Feldmann and his colleagues (Feldmann et al., 1974; Konttinen and Feldmann, 1977) proposed that a "suppressor T cell" elaborates an antigen-specific soluble T cell mediator that focuses antigen on the macrophage membrane in a configuration optimal for the stimulation of B cells to produce antibody. They presented a model which utilizes the T cell mediator to explain mechanistically the phenomenon of antigenic competition. Their T-cell produced molecule was termed IgT, which they proposed was produced during the response to the first antigen. This IgT saturated the available sites on the macrophage membranes. When a second population of IgT, antigen specific mediators, are produced in response to stimulation by a second antigen their action is precluded because available IgT recep-
tors on the macrophage surface are saturated with the IgT mediators produced against the first antigen. This theory is presently under critical scrutiny since during antigenic competition the antibody response to the second antigen is usually initiated in a normal fashion but is terminated prematurely as it comes under the influence of the suppressor T cells or the mediators stimulated by the first antigen. If the model proposed by Feldmann possess merit, the IgT mediators produced in response to the first antigen should block the initiation of the antibody response to the second antigen since the macrophage membranes are saturated by the IgT that is induced by the first antigen. A second line of evidence that detracted from Feldmann's proposed mechanisms was data that showed spleen cells harvested from mice manifesting antigenic competition produced normal responses in vitro to an unrelated antigen which required macrophage cooperation.

Thomas et al (1975) reported that antigen stimulated suppressor T cells released a protein mediator that terminated antibody responses to unrelated antigens when spleen cells were assayed in vitro. The suppressor mediator they described was distinguishable from a second mediator which appeared to provide a helper function and they suggested the suppressor factor may be modulating cell-interactions during antigenic competition. In a more recent study Raff and Hinriches (1977) reported that an anti-thymocyte serum sensitive T-lymphocyte population produced soluble mediator(s) which is absorbed to macrophages. The absorbed mediator directed the macrophage to produce a second mediator that suppressed lymphocyte responses
against unrelated antigens.

Quite possibly, suppressor T cells and/or their products could act on any one or all of the lymphoid cells which cooperate during the initiation of the immune response. During the primary antibody responses, macrophages, helper T cells, and possibly the responding B cells could be the targets of T cell suppression while during cell-mediated immune responses, macrophages and/or the effector or helper T cells could be the target.

Some experimental data show that antibody responses to injected antigens in animals undergoing systemic graft-versus-host reactions are suppressed if the test antigen is administered at the appropriate stage of the graft-versus-host reaction (Katz, 1972, 1974; Liacopoulos and Ben-Efraim, 1975). This form of antigenic competition is 'pseudoclassical' in the sense that it is not mediated by the host T cells, but by T cells in the grafted lymphoid tissues stimulated to react with host histocompatibility antigens, which appears to result in the elaboration of "soluble" suppressor factors which dampen the response to unrelated antigens artificially administered (allogenic effect).

At this time in the murine system it appears that the immature, spleen-seeking T-1 cells play an important role in antigenic competition and soluble mediators (products of T-1 cells) appear to be involved.
Antigen-Specific Suppression. This section will be limited to a discussion of recent advances with respect to studies done on antigen-specific suppression of the immune responses by suppressor T cells. Antigen-induced suppressor T cell activity is with the exception of antigenic competition, specific for the inducing antigen, and exerts no significant suppressive effect on the immune responses to unrelated antigens. The ability of antigen-specific suppressor T cells to distinguish between two related antigens has been well documented. For example, in mice, induction of suppressor T cells to human serum albumin (HSA) will not exert a suppressive effect on the immune response to rabbit serum albumin (Zan-Bar et al, 1975).

Second, horse gamma globulin-induced suppressor T cells suppress the antibody response to the inducing gamma globulin, but not to BGG (Benjamin, 1975).

Antigen-specific suppressor T cell activity is usually assessed by combining lymphoid cells from the animals primed with antigen with an immunogenic form of the antigen or hapten conjugate of the antigen, and comparing the resultant immune responses to those of normal immunocompetent cells along. Data obtained from experiments of this kind show that suppressor T cells inhibit the development of primary antibody responses by normal lymphoid cells in all systems studied thus far. Addition of excess "normal" T cells to antigen-suppressed animals does not restore immune responsiveness to that antigen (Gershon and Kondo, 1970). In fact Lawerence and Weigle (1976) reported that when excess numbers of normal thymocytes are adoptively
transferred into recipients, the subsequent response to 2,4-dinitrophenyl (DNP) is suppressed. They showed that an optimum anti-hapten response was obtained when $10 \times 10^7$ normal thymocytes were infused, but the response was completely abolished when $20 \times 10^7$ normal thymocytes were transferred. The suppressor activity appeared to be associated with T cells and was active in both the cortisone-resistant and cortisone-sensitive thymocyte populations, but suppressor activity was eliminated when the cells were harvested from donors subjected to 1000 rads of X-irradiation.

The antigen-specific T cell populations could be isolated from spleen lymphocyte populations in all systems studied thus far. Suppressor T cells could also be recovered from thymocyte populations (Tada, 1974; Kapp, et al, 1975; HA and Waksman, 1973), but they were not recovered from thoracic duct drainage (Basten et al, 1975). They could also be recovered from lymph nodes, but usually in lower numbers than in the spleen (Kapp et al, 1975). Non-antigen-specific T cells were found to exhibit approximately the same tissue distribution when compared to the antigen-specific suppressor cells.

The mediators of antigen-specific suppression have been thoroughly studied in the elegant experiments of Tada (1974, 1975, 1976, 1977). Tada and his colleagues demonstrated that suppressor T cells collected from ascaris-primed rat can abruptly suppress ongoing IgE antibody responses when infused into recipients. They also showed that carrier-primed mouse thymocytes can inhibit the antibody response to the hapten DNP when they were infused into animals.
subsequently immunized with DNP conjugated to test carrier (in most instances, the carrier was keyhole limpet hemocyanin, KLH). As a result of their efforts to identify the suppressive substance, they showed that cell-free extracts prepared by mechanical disruption of thymocytes or spleen cells, which have suppressor T cells, exerted the same level of suppression as whole cells. The active moiety in the cell extract was found to be a protein with a molecular weight estimated by Sephadex gel filtration to be in the range of 35,000 to 60,000 daltons. Suppressor factor activity could be absorbed by carrier immobilized on immunoabsorbents. The factor is not an immunoglobulin since the suppressive activity could not be removed from thymocyte extracts by anti-immunoglobulin, anti-Fab, anti-u-chain, or anti- \( \gamma \)-chain antibody. The suppressor activity cannot cross histocompatibility barriers since the factor will only suppresses the antibody response in syngenic animals. From the data, Tada suggested that the active component was not an antibody molecule in the classic sense, but may have an antigen-specific reactive site (carrier specificity).

One of the more surprising findings that evolved from the studies discussed above was that the T-cell suppressive factor may be a product of an I-region gene (Tada et al, 1975). Tada et al (1976) showed that suppressor activity could be removed from thymus extract by absorption with alloantibody directed against antigens encoded by the I-region of the H-2 complex of the donor mouse strain. More recently, they presented evidence with indicated that determinants controlled by a locus (Ia-4) that maps in the I-J region of mouse H-2 major histocompatibility complex are selectively expressed
on the antigen-specific suppressor T cells (Tada et al, 1976). Their findings were confirmed by the studies of Murphy et al (1976) and Okumura et al (1976). They showed that the cell type which carries the I-J determinant is involved in the allotype- and idiootype-suppressions respectively. In all the preceding studies the suppressor activity of suppressor T cells and Suppressive factor was removed by anti-I-J antisera. Further functional and serological analysis studies have tentatively identified antigen-specific suppressor T cells as I-J⁺, Ly 2⁺, 3⁺ and Fe receptors T cells.

With respect to the mode of action of the suppressive factor, little is known at present. Kapp et al (1974, 1976) in their studies in which the synthetic terpolymer antigen system composed of L-glutamic acid⁶⁰-L-alanine³⁰-L-tyrosine¹⁰ (GAT) defined the role of genetic control for the production of the immunosuppressive factor(s). Their findings are similar to that of Tada. They postulated a two step mechanism for the mode of action of the suppressive factor on the immune response (Waltenbaugh et al, 1977). The first proposed step, antigen mediated, would result in the production of an antigen-specific suppressive factor and during the second step, the factor mediates a suppressive effect on a target cell. In this model, two T cell populations are involved; one population produces the factor and this factor acts upon a second distinct subsets of T cells which then become the effector suppressor T cells. Thus, activation of a limited number of factor producing cells would result in the generation of a greater number of effector suppressor T cells. However,
further experimentation are required to prove or disprove this model.

Effect of short term Adult Thymectomy. The effect short term adult thymectomy exerts on immunological responsiveness have not been investigated fully. In the murine system, adult thymectomy depletes T-1 cells from the spleen within 2-4 weeks. Murine spleen cells from these animals when assayed after two weeks post-thymectomy show an enhanced DNA synthetic responses in the mixed lymphocyte reaction (Mosier and Cantor, 1971). Additional studies showed that adult thymectomized mice develop enhanced antibody response to T cell-independent antigens such as polyvinyl pyrrolidone (Rotter and Trainin, 1974) and Corynebacterium parvum (Howard et al, 1973). Rotter and Trainin (1974) showed that the enhanced antibody responses can be suppressed by infusing the thymectomized mice with syngeneu thymocytes from young donors. Kappler et al (1974) in their studies concerned with functional heterogeneity among thymus derived lymphocytes of the mouse showed that thymectomized animals have increased helper T cell activity for primary responses to trinitro-phenylated (TNP-SRBC) erythrocytes and sheep red blood cells. With respect to the rabbit system, Taniguchi and Tada (1974) clearly demonstrated that the relative diminution of T cells, caused either by thymectomy in early adult-hood or by treatment with anti-lymphocyte serum, significantly enhanced the antibody response to dinitro-phenylated-bovine gamma globulin (DNP-BGG). Furthermore, the enhancement was associated with a marked increase in affinity for the hapten by the antibody produced. Previous studies in their laboratory (Okamura and Tada, 1971) using
the rat system have showed that adult thymectomy markedly enhanced and prolonged their production of IgE homocytotrophic antibody against DNP-ascarids. The absence of T suppressor cells, depleted by short term adult thymectomy, may have allowed the elevated IgE response to persist in these animals, which perhaps retained sufficient numbers of helper T cells.

The effect of adult thymectomy of the mouse on the secondary immune response, appears to be far-reaching since thymectomized mice cannot be primed effectively for memory-helper T cell activity or for the expression of delayed hypersensitivity reactions or secondary cytotoxic lymphocytes responses (Kappler et al, 1974; Simpson and Cantor, 1975). These findings appeared to be contradictory to the experimental results reported in this dissertation and shall be dealt with in the discussion.

More recent experiments have shown that suppressor, helper, and cytotoxic T cell activity could be differentially regulated by injection of complete Freund's adjuvant (CFA). Reinisch et al (1976) reported that after the injection of CFA, both helper and suppressor activity increased, whereas cytotoxic activity (K cell) diminished markedly. They then treated sham or thymectomized animals with adjuvant and subsequently challenged them with either allogenic tumor cells or murine sarcoma virus.

Thymectomy abrogated the induction of suppressor cell activity in vitro (Reinisch et al, 1977). Despite the administration of complete Freund's adjuvant, thymectomized animals developed a normal
cytotoxic immune response while sham-thymectomized animals showed no cytotoxic activity and these animals subsequently died of progressive tumors, whereas tumors in the thymectomized animals regressed.

Although further experimentation is required for positive identification of the types of T cells that are depleted after adult thymectomy, it might be safe to suggest that during adult life the thymus produces and maintains a population of short-lived T cells in the peripheral lymphoid tissue such as the spleen and this short-lived cell exerts a regulatory function during the development of both humoral and cell-mediated immune responses, and possible influences the development of immunological memory.

Effect of antilymphocyte serum. Antilymphocyte serum (ALS) is produced by immunization of heterologous species with lymphoid cells or the solubilized membrane fractions. ALS can be raised by immunization with lymphocytes from most lymphoid tissues such as thymus, lymph nodes, spleens, peripheral blood leukocytes (Lance et al, 1973).

In vivo injection of ATS will depress the humoral antibody response to a variety of antigens, presumably by depleting recirculating T cells which may exert T cell helper functions (Lance, et al, 1973). Baum et al (1969) reported that rats pretreated with IgG fraction of ALS showed a marked suppression of the antibody response to SRBC, however, the response to KLH was sixteen times greater than that of non-treated animals. Similar results were obtained in
the studies by Kerbel and Eidinger (1971) when using ALS treatment or ALS treatment in conjunction with adult thymectomy. In subsequent studies, it was shown that after administration of single dose of ALS, antibody responses to some T cell-independent antigens such as pneumococcal polysaccharide (Baker et al, 1970a, b; 1974a; Barthold et al, 1973) and polyvinyl pyrrolidone (Kerbel and Eidinger, 1972) are often markedly enhanced. ALS may cause depletion or inactivation of a suppressor T cell since the enhanced responses to T cell-independent antigens was reduced to threshold response levels after the injection of syngeneic thymocytes into ALS-treated animals (Baker et al, 1970b, 1974). Further evidence to support this hypothesis has come from experiments in congenitally athymic (nu/nu) mice whose response to pneumococcal polysaccharide was not affected by ALS treatment (Baker et al, 1973). However, more recently, Warr et al (1975) showed that although antibody response to pneumococcal polysaccharide may be enhanced after administration of ALS, the enhanced responses are not reduced by injection of normal syngeneic thymocytes. These results do not correlate with data described above and cannot be explained as of yet. It is conceivable that the enhanced responses to T cell-independent antigens after ALS treatment may not be due to a depletion of suppressor T cells, but to a mitogenic effect of ALS on B cells (Pierce and Kapp, 1976). More experimentation is needed to resolve this paradox.
MATERIAL AND METHODS

Experimental Animals

Rabbits. Randomly bred New Zealand White rabbits weighing 1.5-1.8 kilograms (8-10 weeks old) were used. They were obtained from a closed colony maintained in this laboratory. The rabbits were fed Purina rabbit pellets and given water ad libitum.

Sheep. An adult female sheep was maintained at the LSU disease animal research center and was used as red blood cell donor. Sheep blood was collected monthly from the jugular vein, into an equal volume of chilled Alsever's solution (Campbell et al, 1970) and stored at 4\(^\circ\) C until needed.

Horse. Two Welsh ponies were immunized with rabbit thymocytes to produce anti-rabbit thymocyte serum. They were kept in the LSU disease animal research center.

Antigen

Preparation of Dinitro-phenylated proteins. The 1-fluoro-2,4-dinitrobenzene (DNFB) was purchased from Eastman Organic Chemicals, Rochester, N.Y. and was recrystallized from absolute ethanol three
times before use. Hen ovalbumin (OVA), Bovine gamma-globulin (BGG),
Bovine Serum albumin (BSA), were obtained from Sigma Chemicals,
St. Louis, MO as Cohn fraction V. Keyhole limpet hemocyanin was
purchased from Calbiochemicals, San Diego, CA. in recrystallized
powder form.

The 2,4-dinitro-phenylated proteins were prepared by the
method of Eisen (1964). One gram of DNFB was dissolved in 50 ml
glass of distilled, deionized water in an erlenmeyer flask wrapped
with aluminum foil to shield the contents from light. One gram of
Potassium Carbonate was added and then one gram of protein was added
after the salt dissolved, and the pH approached 9. The resulting
solution was stirred in the dark for 24 hours at 37° C. After this
period the DNP-conjugated protein was extensively dialyzed in the
cold against phosphate buffer saline (PBS), pH 7.3. The protein con­
tent was determined by dry weight determination and the dry weights
so obtained were corrected for the presence of PBS. The degree of
DNP substitution was estimated spectrophotometrically by assuming
that all hapten groups (DNP) were coupled to ε-amino groups of lysine.
The molar absorbancy of free ε-DNP-L-lysine was 17,530 at 360 nm
(Eisen, 1964). The formula for determination of numbers of sub­
titution of DNP into protein is as follows:

\[
\text{Optical density of DNP-protein conjugate} \quad \frac{\text{# of substitution}}{\text{Molarity of conjugate}} = \frac{\text{E-DNP-L-lysine molar extinction coeff.}}{E-DNP-L-lysine molar extinction coeff.}
\]
where:

Optical density of DNP-protein conjugate = absorbance of DNP-protein conjugate at 360 μm E-DNP-L-lysine molar extinction coefficient = 17530. The following hapten-protein conjugate were prepared: DNP$_7$-OVA, DNP$_{21}$-BSA, DNP$_{28}$-BGG, DNP$_{530}$-KLH. Subscripts refer to the average number of moles of DNP per moles of protein. The immunization schedules used are described below.

Collection and preparation of rabbit serum. Blood was collected from the central ear artery and allowed to clot at room temperature for one hour, then freed from the tube by ringing the clot and stored at 4° C to promote clot retraction. The serum was decanted and clarified by centrifugation and stored at 20° C until serological assays were done.

Coupling of antigen to sheep erythrocytes by the Chromium Chloride method. The chromium chloride method for coupling antigen to erythrocytes is widely used (Gold and Fudenberg, 1967; Faulk and Houba, 1973; Kofler and Wick, 1977). This method is relatively simple, in that no special reagents or difficult manipulations are required in this procedure. Although there are reports (Goding, 1976) which suggested poor reproducibility due to some variables which may affect the coupling process, in our experiments no such problems were encountered. There exists only limited information about the coupling mechanism itself. Faulk and Houba (1973) suggested that
chromium chloride may activate the carboxyl residues in the protein of the red blood cell membrane and hence create active sites for antigen binding.

In our laboratory preliminary experiments were run to determine the concentrations of protein-antigen, chromium chloride and sheep erythrocytes required for optimal coupling. The procedure we adopted for optimal coupling is as follows. To 0.5 ml of packed SRBC which had been washed three times in cold saline was added 0.5 ml of protein antigen at a concentration of 3 mg per ml. Immediately 0.5 ml of 0.1% chromium chloride was added to the mixture and the resulting mixture was shaken manually. The mixture was held at room temperature for 6 minutes with occasional stirring. The coupling reaction was stopped by the addition of 10 ml of cold phosphate buffer saline, pH 7.3. After three washes with cold saline, the antigen-coupled SRBC were ready to be diluted and dispensed into microtiter plates for the passive hemagglutination assay. A concentration of 1.0% sensitized SRBC was found to be required for optimal agglutination in the presence of anti-DNP antiserum. During the coupling process, the concentration of antigen used is important. Excess antigen may inactivate chromium chloride which could prevent creation of active sites on the erythrocyte membrane. On the other hand, antigen concentrations that are too low in relation to the chromium chloride concentration may result in incomplete filling of the erythrocyte binding sites and which could result in spontaneous agglutination.
Table 1. Hank's balanced salt solution used as diluent in passive hemagglutination assays.

<table>
<thead>
<tr>
<th>Constituents</th>
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Flask 1. NaCl, 8g; KCl, 0.4g; Na$_2$HPO$_4$.7H$_2$O, 0.189g; KH$_2$PO$_4$, 0.05g; Glucose 2.0g; Gelatin, 1.0g in 700 ml of boiled redistilled water.

Flask 2. CaCl$_2$.2H$_2$O, 0.265g in 100 ml boiled redistilled water.

Flask 3. MgSO$_4$, 0.09772g in 100 ml boiled redistilled water.

Flask 4. NaHCO$_3$, 0.34g in 100 ml boiled redistilled water.

Procedure

1. Autoclave all four flasks for 10 minutes. Store at refrigerator temperature.

2. When needed, mix the contents of flasks 2, 3 and 4 into the contents of flask 1.

3. Check pH (range 7.2 to 7.6).
Passive hemagglutination assay. Serum collected from pre-bleed and immunized rabbits were absorbed three times with equal volumes of packed SRBC. The sera were then heat-inactivated (56°C, 30 min) prior to use. Microtiter techniques and equipment (Cooke Engineering Co., Alexandria, VA) were used to run the agglutination titers. Microtiter plates with V-shaped wells were loaded using a microtiter pipette with 0.05 ml of Hank's balanced salt solution (HBSS). Five-hundredth of one ml microdiluters were cleaned, calibrated, and loaded with serum. The antiseraum (0.05 ml) was delivered to the first well and mixed with the HBSS diluent by a rotating action. Serial dilutions were made through at least 12 wells and sometimes 15-16 wells, as the titer dictated. Next 0.05 ml of 1% antigen-coupled SRBC suspension was added to each well and mixed by gentle tapping of the plate edge. To control for spontaneous agglutination, the last well of each plate contained diluent and antigen-coupled SRBC only. The titer was recorded as the reciprocal of the highest dilution showing agglutination.

Preparation of immunoabsorbents. The procedure used was essentially the same as that recommended by Pharmacia Chemicals (Piscataway, NJ). Twenty grams wet weight of Sepharose 4B was washed on a sintered-glass funnel with distilled water. The washed Sepharose was suspended in 50 ml of water and 2 grams of solid cyanogen bromide was added to the suspension while it was being stirred. The activation procedure was done in a well-ventilated hood. The pH of the
suspension was kept at pH 11 by periodic addition of 2 M NaOH solution. The reaction was terminated by the addition of cold water directly to the mixture when the pH remained constant. The mixture was then transferred to the sintered-glass funnel and washed with cold 0.1 M NaHCO₃ several times (approximately 300 ml). The activated Sepharose was added to 50 ml of normal or immunized immunoglobulin at a concentration of 3 mg per ml in 0.1 M NaHCO₃. The suspension was stirred for 18 hours at 4° C and then washed with phosphate buffered saline, pH 7.2, until no absorbance at 280 nm could be detected in the filtrate. The efficiency of coupling was determined by measuring the absorbance of the protein solution before and after coupling to activated sepharose. Next the immunoabsorbent was suspended in 3 M NaSCN for 1 hour to make sure that the protein coupled to the sepharose was not released from the immunoabsorbent under these conditions, since 3 M NaSCN was used to elute antigen fragments from the coupled-antibody in the immunoabsorbent.

**Thymectomy.** Rabbits which weighed between 1000 and 1500 grams (approximately 6 to 8 weeks old) were used. At this age, there is limited vascularization of the chest dermis and little or no fat permeating the thymus gland. The rabbits were injected with 0.4 ml of Combiotic intramuscularly 24 hours before thymectomy. The chest area was shaved from the neck to the epiphisternum, and cleaned with PhisoHex and water. Initially 0.3 ml of 2.5% sodium pentabarbital was administered intravenously and additional anesthetic was given
during the 20 to 30 minutes surgical period as needed. An incision was made through the dermis and muscle layers to the left of the sternum. The chest cavity was entered by cutting through the first and second ribs at their sternocostal junctions. The muscle layers were cut to the third rib and the opening was retracted using stainless steel spreaders. By gentle pulling, the thymus was separated from its attachment site on the aorta in toto with dog-toothed forceps. The musculature and ribs were closed with a single suture line of 0000 gut silk or synthetic suture. The rabbits were housed individually in steam-cleaned cages and maintained with purina pellets and water with Terramycin for at least one week before experimental manipulation.

Preparation of thymus and spleen cell suspensions and their extract. Spleens and thymuses were removed from normal or hyperimmunized rabbits and placed in a small amount of chilled Medium 199 (Grand Island Biologicals, NY). They were minced with forceps and scissors and released from fibrous connective tissue by gentle shaking after the minced tissue was delivered to a screw-capped conical centrifuge tube. Next the cells were passed through a #74 stainless steel sieve, washed three times with cold Medium 199, respended in phosphate buffered saline, pH 7.2, and after total nucleated cell counts were made the numbers were adjusted to $2 \times 10^9$ cells per ml for thymus and $4 \times 10^8$ cells per ml for spleen.
Cell-free extracts were prepared from thymocytes and spleen cells by sonication. The cell suspensions were subjected to sonication in a Branson sonicator (Branson, Danbury, CT) with 20 kc/sec for 5 minutes while in a vessel immersed in ice water so as to minimize the rise in temperature. The sonicated suspensions were then centrifuged at 20000 x g for one hour and cell-free supernatant fluids were collected and stored in -20° C.

Adsorption studies. The thymocyte extract was divided in four or five aliquots and absorbed with differ-immunoabsorbents. They were absorbed with IgG fraction of anti-rabbit thymocyte antisera, normal horse IgG, or ovalbumin immobilized on Sepharose in an immuno-absorbent column. The immunoabsorbent columns were constructed by adding protein-coupled Sepharose 4B to a 10 ml plastic syringe fitted with glass wool at the outlet. Two ml of thymocyte extract (4 x 10^9 whole cell equivalent) was added to the column and incubated at 4° C for 3 hours. After the incubation period, the column was washed by passing 50 ml of phosphate buffer saline, pH 7.2 through the immuno-absorbent to remove those constituents of the extract that were not specifically bound to the immunoabsorbent. The eluate was monitored at 280 nm for the estimation of protein content. To elute the fractions that were bound specifically to the immunoabsorbent column, 3 M NaSCN in phosphate buffer saline, pH 7.2 was passed through the column and the eluates were collected and were dialyzed against three changes of phosphate buffer saline to remove the NaSCN. Before the
fractions were injected into the rabbit, the fractions which were to be assayed were adjusted to the starting volume of whole extract by concentrating the dialyzed eluant with a vacuum ultrafiltration apparatus.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of thymus extract (SDS-PAGE).** The thymus extracts that were eluted from the immunoabsorbent columns with 3 M NaSCN were dialyzed against three changes of deionized and distilled water. The dialyzed extracts were freeze-dried in 1 ml of saline. SDS-Page was run according to the method of Laemmli (1970) as modified by V. Barta (personal communication). Stock reagents for polymerization of the gels were prepared as in Table II. The sample gels (resolving gels) were polymerized in a glass template by light exposure for 20 to 40 minutes and overlaid with 3 to 4 mm of water on top of the sample gel mixture until the gel polymerized. After polymerization was completed, the water was removed and with the template in an upright position the stacking gel mixture (Table I) was added on top of the resolving gel with a water overlay. A plastic well-maker was placed on top of the spacer gel. The gels were placed at a distance at about 30 cm from the photopolymerizing light source and after 10 to 15 minutes of exposure, the light source was moved up to the column for an additional 15 minutes which ensured gradual polymerization and a gel with homogeneous pore size.
Table II. Dissociation buffer for protein samples in SDS-PAGE 2X buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M Tris-HCl (pH 6.8)</td>
<td>50 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>4g</td>
<td>2g</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>10 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>glycerol</td>
<td>10 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>0.01% Bromophenol blue solution</td>
<td>20 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>10 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>100 ml</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Staining solution for gel:
1. Dissolve 0.275g of coomassie brilliant blue in 100 ml of methanol: water mixture (1:1 v/v).
2. Stored at room temperature.
3. Just before use, add 10 ml of glacial acetic acid per 100 ml of dye solution.
4. Stain for 2 hours at room temperature.

Destaining solution for gel:
1. Prepare 10% acetic acid and 30% methanol in distilled water.
2. Destain for 48 hours with three changes of the destaining solution.

For storage of the gel:
1. Stored in 3.0% acetic acid in distilled water.
One-half milliliter of each of appropriate thymocyte extracts were added to 0.5 ml of 2X dissociation buffer. The mixture was heated to 100°C for 2 minutes with constant shaking and then cooled at 4°C in a refrigerator. Electrophoresis was performed in a Savant Gel electrophoresis unit (Savant Instruments, New York). Fifty microliters of the boiled extracts were placed in the wells on top of the spacer gel with Eppendorf pipets. Then the samples were overlaid with electrode buffer and electrophoresed at 30 milliamperes until the tracking dye was approximately 0.5 cm. from the bottom of the gel. The electrophoresis process took approximately 3 hours. After electrophoresis, the gels were removed from the glass template by gently forcing staining solution (Table III) between the gel and the glass plate with a syringe. The gels were then stained for two hours at room temperature and destained for 48 hours at 60°C with each electrophoresis, a set of molecular weight marker (standard) manufactured by Biorad (San Diego, CA) was run with the sample, these were used as molecular weight calibrations for the sample assayed.

Preparation of horse anti-rabbit thymus serum (HARTS). The antiserum was from the same pool collected and prepared by Arnold (1975). Two Welsh ponies were immunized three times by intravenous injections of $3 \times 10^{10}$ New Zealand White rabbit thymus cells suspended in 30 ml of HBSS (DeLaNoue et al, 1972) at weekly intervals. Two liters of blood were taken from the jugular vein on the second, third, and fifth week after the final immunization. The serum was recovered
Table III. Components for the preparation of resolving (sample) gel for SDS-PAGE.

<table>
<thead>
<tr>
<th>Components for the preparation of resolving (sample) gel for SDS-PAGE</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
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*TEMED - use undiluted

Components for the preparation of spacer for SDS-PAGE

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*TEMED - use undiluted
as previously described for rabbit serum and stored in 50 ml aliquots at -20° C.

**DEAE fractionation of HARTS.** In order to avoid provoking serum sickness in rabbits which were injected with HARTS, the IgG fraction of the horse antiserum anti-thymus gamma globulin (ATGG) was used. Thirty grams of DEAE Whatman DE 23 (Cat. # 24233) was suspended in 800 ml of 0.5 N NaOH and 0.5 N NaCl. The gel slurry was allowed to settle for 30 minutes. The supernatant fluids, which contained the fine particles were decanted and the DEAE was washed with 600 ml of 1 N NaCl. This process was repeated a second time and then the DEAE was filtered through two thickness of 15 cm Whatman #1 qualitative filter paper under negative pressure. Next, 400 ml of 1 N HCl was added to the slurry and immediately filtered. The DEAE was washed with distilled water and stored at 4° C overnight. The DEAE was next suspended in 1 liter of 0.01 M phosphate buffer, pH 8.0. The slurry was filtered and washed with phosphate buffer until the pH approached 8.0, at which time it was stirred under negative pressure to remove trapped air. The DEAE was packed into a glass column (20 x 400 cm) by pouring the gel slurry into the column followed by passing 200 ml of phosphate buffer through the column.

A 40 ml aliquot of HARTS was heated to inactivate complement (56° C, 30 min) and then absorbed with thrice washed rabbit RBC (0.2 ml packed RBC per 1.0 ml serum) at room temperature for 30 minutes to remove anti-rabbit hemolysin. The absorbed serum was dialyzed
in the cold against 100 volumes of 0.01 M phosphate buffer, pH 8.0 which was changed three times at 8 hour intervals. Next, the dialyzed serum was centrifuged at 5000 rpm for 30 min. to remove pseudoglobulin precipitate.

An aliquot of 10 ml of dialyzed HARTS was added to the packed DEAE column. The serum was allowed to enter the bed, and then the IgG fraction was eluted with 0.01 M phosphate buffer, pH 8.0. The eluant was collected in 10 ml. fractions and their protein content was estimated by measuring optical density readings at 280 nm in a Beckman double beam spectrophotometer. Fractions which possessed an optical density of 0.5 or greater were pooled. Depending upon the length of the column, the IgG peak usually started after 50 ml had been collected and was generally completed within the next 30 ml. The pooled fractions were concentrated to 5 ml using polyvinyl pyrrolidone or Aquacide II (Calbiochem, San Diego, CA) at 4° C.

Total protein determinations were performed on the concentrated IgG fractions by a modification of the Lowry method (Lowry et al, 1951). The total concentration of each ATGG preparation was adjusted to 10 mg per ml with the addition of 0.15 M NaCl.

Normal horse gamma globulin (NHGG) was prepared from normal horse serum in the same manner as described for HARTS.

The purity of ATGG and NHGG was assayed by the immunno-electrophoresis method as described by Arnold (1975). The results indicated that the IgG fractions are relatively free of contaminating serum proteins as only one single band was present in the immun-
electrophoresed sample when reacted with anti-whole-horse-serum prepared in rabbits.
Figure 1. The experimental design for the studies of the effect of the ATGG-treatment on the antigenic competition-induced suppression of the anti-DNP response.
1. 1° injection; DNP-OVA
2. Free carrier; BGG, KLH (in FCA)
3. 2° injection; DNP-OVA, DNP-BGG, DNP-KLH
4. Horse anti-rabbit thymocyte gamma-globulin (ATGG)
5. Titer anti-DNP Ab by PHA (CrCl₃ modified method of Fudenberg et al.)
Figure 2. The experimental protocol for the soluble thymus extract studies.
THYMUS

EXTRACT (2 x 10^9 cell equivalent)

IMMUNOABSORBENTS - Sepharose 4B

- ATGG
- NHIG
- OVA

0.15 M PBS eluted fractions

10 mg DNP-OVA INFUSE RABBITS

PHA anti-DNP assay

3 M NaSCN eluted fractions

DIALYSIS vs. Saline, Water

FREEZE-DRIED recostitute to 1.0 ml

SDS-PAGE
INTRODUCTION

It has generally been accepted that thymus-derived cells (T cells) play different roles in the homeostatic regulation of immune responses (Reviewed in (1)). In addition to the well-established helper functions, a vast body of informations has also pointed to active suppressor mechanisms on both T-dependent and T-independent antigens mediated by suppressor T cells (2-6). In mice, suppressor T cells have been shown to be a distinct population from amplifier T cells and to differ significantly with respect to types of surface antigens present, tissue distribution, radiosensitivity and access to ALS (7-9). In a different system, the rabbit, studies with adult thymectomy and ALS treatment have reported an increase in primary humoral response to T-dependent antigens in a short term study (10). These results suggested rabbit T cells possess an important regulatory role in the immune response, reminiscent of mice.

In the experiments reported here, we have utilized antigenic competition, which has been thought to be mediated by suppressor T cells in the mouse system (11-12), as the system to examine the effects of adult thymectomy and ATGG treatment on the primary and secondary immune responses in the rabbit in vivo. We wish to report that adult thymectomy and ATGG treatment shortly before immunization with T-dependent antigen markedly enhanced the level of humoral antibody synthesis and relieved the unresponsive state seen in antigenic competition between heterologous carriers.
MATERIALS AND METHODS

Animals: Eight to ten week old male and female New Zealand White rabbits weighing between 1500 and 1800 g were used throughout this study. The animals were obtained from a colony maintained by our laboratory. Experimental groups were comprised of at least 4 rabbits.

Antigens: The antigens used were ovalbumin (OVA), Bovine gamma-globulin (BGG), Bovine serum albumin (BSA), and Keyhole limpet hemocyanin (KLH). These antigens were dinitrophenylated according to the method of Eisen (13).

Rabbit Immunization: The immunization schedule of Katz et al (14) was employed. For primary immunization, rabbits were injected intravenously via the marginal ear vein with 3.0 mg of DNP_8-OVA daily for three successive days. One week later, a supplemental immunization with 50 ug of BGG or 50 ug each of BGG and KLH was injected subcutaneously. Five weeks after the primary immunization, 5 mg of DNP_8-OVA or DNP_28-BGG in saline, were injected intraperitoneally (the secondary immunization). Modification of the immunization protocol will be described in the results section.

Horse anti-rabbit thymocyte gamma globulin (HATGG): A Welsh pony was injected for three successive weeks with $3 \times 10^{10}$ rabbit thymocytes suspended in 50 ml Hanks balanced salt solution HBSS according to
the method of DeLaNove et al (15). The pony was test bled 2 or 3 weeks after the last injection of thymocytes and exsanguinated on the fifth week. The IgG fraction (HATGG) was separated from elsewhere (16). The HATGG fractions were adjusted to 10 mg protein per ml with phosphate buffer (PB) and cytotoxic indices were done with this preparation by the due exclusion method as described by DeLaNova et al (15). Normal horse gamma globulin (NHGG) was obtained from non-immune horse serum in the same manner as described for HATGG. Experimental rabbits received intravenous infactions of HATGG or NHGG in 30 mg doses.

Thymectomy: Young adult rabbits from selected litters at 1 kg body weight were thymectomized under anesthesia with pentobarbital. The thymus was removed in toto through a median sternotomy. Thymectomized rabbits were allowed to convalesce at least 7 days prior to further experimental manipulation.

Adsorption of HATGG: Ninty mg of HATGG in approximately 3 ml PB was adsorbed three times with 0.4 ml viable rabbit thymocytes (approximately 9 x 10^8 thymocytes). Each adsorption step was carried out in a 37° C water bath for 30 minutes.

Immunoabsorbents: Globulin fractions of normal rabbit sera were coupled to CNBR activated Sepharose 4B by mixing the activated beads with the dialyzed globulin fraction in coupling buffer for 24 hours at 4° C. After the coupling procedure, the beads were washed extensively with PBS until no protein could be detected in the eluates. The immunoabsorbent columns were prepared by
packing required quantities of beads into barrels of 5 ml syringes. HATGG appropriately diluted in PBS was applied to the column, reacted at 4° C for 3 hours and then eluted with PBS. The HATGG eluates were then injected into the experimental rabbits in 30 mg doses.

Anti-DNP antibody assay: Sera were collected from experimental animals at various intervals as described in the result sections. Anti-DNP titers were done by the standard PHA microtiter technique using DNP_{21}-BSA conjugated sheep red blood cells (SRBC) as test antigens. DNP_{21}-BSA was conjugated to SRBC by the chromium chloride method described elsewhere (17).
RESULTS

General Considerations

Preliminary experiments were performed to determine the optimal concentration of antigen, DNP-OVA, required to produce a detectable anti-DNP primary immune response. Figure 3 details the primary immune response in vivo for four different groups of rabbits which had received 3 mg, 5 mg, 10 mg, 20 mg of DNP-OVA respectively for 3 consecutive days. As shown in the figure, 3 mg of DNP-OVA administered for 3 consecutive days was a sub-optimal dosage in that no detectable humoral antibody was detected by the PHA method through 21 days postimmunization. Antigen doses of 5, 10 or 20 mg of DNP-OVA for 3 consecutive days produced significant antibody responses by the 15th day. We selected for subsequent experiments, 10 mg of DNP-OVA (3x) as the optimal dose for primary immunization and 3 mg of DNP-OVA (3x) for the sub-optimal dose for primary immunization of rabbits in vivo.

Effects of ATGG treatment or adult thymectomy on the anti-DNP response.

In these experiments, the effect of ATGG treatment or adult thymectomy on the short term anti-DNP primary responses was investigated. Figure 4 shows the anti DNP responses for 4 groups of rabbits injected with different regimens of ATGG. The first group received 3 injections of 15 mg HATGG, the second group received 3 injections of
Figure 3. Anti-DNP primary antibody response for rabbits immunized with 3, 5, 10, or 20 mg of DNP-OVA for 3 consecutive days. Each curve represents the mean agglutination titer for at least 4 animals at each day of bleeding.
Figure 4. Anti-DNP primary antibody response for rabbits immunized with 3 mg DNP-OVA for three consecutive days (Suboptimal dosage) and treated with ATGG at a dosage of either 15, 30, or 60 mg for 3 days at one day before, same day and one day after the first day of antigen injection. One group of rabbit received 30 mg ATGG at days 6, 7, and 8 after the first antigen injection. Each curve represents the mean agglutination titer for at least 4 animals at each day of bleeding.
ATGG (X 3) 

<table>
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<th>days</th>
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<td>15 mg</td>
<td>-1, 0, 1</td>
<td>Δ—Δ</td>
<td>256</td>
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<tr>
<td>60 mg</td>
<td>-1, 0, 1</td>
<td>Δ—Δ</td>
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ATGG PRIMARY (DNP-OVA) 3 mg
30 mg HATGG and the third group received 3 injections of 60 mg HATGG on day -1, 0, 1 of the first antigen injection with the suboptimal dose of DNP-OVA (3 mg, 3x). A fourth group of rabbits were immunized with suboptimal dosage of DNP-OVA only and received no ATGG treatment. As shown in the figure, only those rabbits which were treated with 30 mg ATGG for 3 days showed a markedly enhanced primary response when compared to control rabbits which received no ATGG treatment.

The results of these experiments suggested that ATGG treatment may enhance the anti-DNP immune response by either eliminating a suppressor activity or augmenting helper activity. We investigated these possibilities further by performing adult thymectomy which had been shown in other systems to increase humoral responses in short term studies (18, 19). Four groups of young rabbits, approximately seven weeks old, were thymectomized. At days 7, 14, 21, 30 days post thymectomy different groups of rabbits were immunized with the suboptimal dose of DNP-OVA (3 mg, 3x). Figure 5 shows the results of this study. A significant enhancement of the primary antibody response to DNP was seen in the experimental group which had been immunized 30 days post thymectomy. The other 3 groups of rabbits showed elevated anti-DNP responses when compared to the control group but were significantly lower than responses in rabbits thymectomized 30 days previously.

Figure 6 summarized the findings on the effects of ATGG treatment and adult thymectomy during the primary response in the rabbit. As shown, if an optimal dose of DNP-OVA was injected into
Figure 5. Anti-DNP primary antibody response for rabbits immunized with 3 mg DNP-OVA for three consecutive days (suboptimal dosage) after 7, 14, 21, or 28 days post thymectomy. Each curve represents the mean agglutination titer for at least 4 animals at each bleeding.
PHA titer (anti-DNP)

thymectomy

-  
7 days  
14 days  
21 days  
28 days  

PHA titer (anti-DNP)

3 mg DNP-OVA  
Primary (days)

50
Figure 6. Anti-DNP primary antibody response for 5 groups of rabbits. Three groups of rabbits were immunized with 3 mg DNP-OVA for three consecutive days either with no further treatment (0---0), or were immunized 30 days after adult thymectomy (———), or treated with 30 mg ATGG for 3 days at one day before, same day, or one day after the first antigen injection (———). Two other groups of rabbits were immunized with 10 mg DNP-OVA for 3 consecutive days (optimal dosage) either with no further treatment (0-----0) or were treated with 30 mg ATGG for 3 consecutive days with the same schedule as mentioned above. Each curve represents the mean agglutination titer for 6-8 animals at each day of bleeding.
DNP-OVA TREATMENT

<table>
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<tr>
<td>3 mg</td>
<td>-</td>
<td>O</td>
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<tr>
<td>3 mg</td>
<td>ATGG</td>
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<tr>
<td>10 mg</td>
<td>ATGG</td>
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Primary Response Days

DNP-OVA
rabbits subsequently treated with 30 mg ATGG, they showed no enhanced anti-DNP titer and was virtually the same as the experimental groups which had received an optimal dose of DNP-OVA alone.

**Effects of ATGG treatment and adult thymectomy on the secondary immune responses**

Antigen-induced suppression (AIS), as seen in the phenomenon antigenic competition, has been suggested to be a T-suppressor cell mediated event. Therefore, if adult thymectomy or ATGG treatment interfere with T-suppressor cell action, the phenomenon AIS could be relieved by such treatment.

Four experimental groups were used in this experiment. Group 1 consisted of animals which were primed with suboptimal doses of DNP-OVA (3 mg, 3x) and five weeks later, they received an intraperitoneal injection of 5 mg of homologous antigen, DNP-OVA (secondary immunization). The results shown on Figure 7 demonstrated a vigorous secondary response to the hapten DNP. The anti-DNP response in this experiment served as control for later experiments.

The next two groups of rabbits were used to demonstrate the carrier effect. One group was immunized with suboptimal dosage of DNP-OVA and 5 weeks later received 5 mg of heterologous antigen DNP-BGG intraperitonealy. The other group was immunized with the same regimen as the previous group except they were supplementally immunized with 50 ug of the carrier BGG in Freund's complete adjuvant by an intramuscular injection at day 7. The latter group of rabbits
Figure 7. Anti-DNP primary and secondary antibody response for rabbits received primary immunization with 3 mg DNP-OVA for 3 consecutive days and received secondary injection with 5 mg DNP-OVA (●—●) or 5 mg DNP-BGG (○—○) ip on day-35 after the first antigen injection. Each curve represents the mean agglutination titer for 6-8 animals at each day of bleeding.
SECONDARY INJECTION

- - DNP-OVA
O--O DNP-BGG

PHA titer (anti-DNP)

Days

DNP-OVA (I°)
3 mg

7 14 21 28 35
PRIMARY

2° INJECTION

4 7 10 15 20
SECONDARY
SECONDARY INJECTION

- • DNP-OVA
- O O DNP-BGG

PHA titer (anti-DNP)

Days

DNP-OVA (1°) 3mg

PRIMARY

SECONDARY

2° INJECTION
produced significant levels of anti-DNP during the secondary response after they were immunized with DNP-BGG (Figure 8).

Antigen-induced suppression was achieved when animals were immunized with suboptimal dosage of DNP-OVA (3 mg, 3x) and at day 7 received 50 ug each of the heterologous carriers BGG and KLH in Freund's complete adjuvant intramuscularly and five weeks later, they received a secondary immunization with 5 mg DNP-BGG intraperitoneally. The secondary anti-DNP antibody response produced was at the same level as the group that had received no supplemental immunization with the carrier (Figure 6).

In an attempt to alleviate the antigen-induced suppression as seen in the previous experiment, one group of rabbits was treated with 30 mg ATGG at day 6, 7, 8 surrounding the supplemental carrier injection after primary immunization with suboptimal dosage of DNP-OVA. This ATGG injection dosage was selected since it had provoked the greatest elevation of primary anti-DNP activity (as seen in Figure 4). Control groups immunized in an identical manner were treated at days 6, 7, 8 with ATGG absorbed with rabbit thymocytes, normal horse gamma globulin or ATGG absorbed with rabbit Ig on an immunoabsorbent column. The resulting secondary anti-DNP responses are shown in Figures 9 and 10, antigen-induced suppression was abrogated in those experimental groups of rabbits treated with ATGG or ATGG absorbed with rabbit Ig. No secondary anti-DNP responses were observed in those experimental groups treated with normal horse gamma globulin or ATGG absorbed with rabbit thymocytes. Interestingly, the primary anti-DNP response which
Figure 8. Anti-DNP primary and secondary antibody response for rabbits immunized with 3 mg DNP-OVA for 3 consecutive days and received no free carrier (Δ--Δ), or supplementally immunized with free carrier, 50 μg BGG (●--●), or 50 μg KLH in FCA (○---○) sc; and immunized with 5 mg DNP-BGG or 5 mg DNP-KLH ip. Each curve represents the mean agglutination titer for 4-8 animals at each day of bleeding.
Figure 9. Anti-DNP primary and secondary antibody response for rabbits primed with 3 mg DNP-OVA for 3 consecutive days, and received no free carrier (Δ------Δ), or supplementally immunized with 50 µg each of BGG+KLH in FCA at day 7 and received either no ATGG treatment (0----------0), or 30 mg ATGG at days 6, 7 and 8 (0---------0) iv, or 30 mg ATGG, which had been adsorbed with rabbit thymocytes, at days 6, 7 and 8 iv. (Δ------Δ) All rabbits were then immunized with 5 mg DNP-BGG ip on day 35 after the first antigen injection. Each curve represents the mean agglutination titer for 4-6 animals at each day of bleeding.
Primary

Secondary

free carrier  ATGG  2nd injection

- - BGG + KLH  67.8  DNP-BGG
- - BGG + KLH  -  DNP-BGG
- -  -  -  DNP-BGG
- - BGG + KLH adsorbed  DNP-BGG

PHA titer (anti-DNP)

Days

DNP-OVA 3mg ATGG

7 14 21 28 35 4 7 10 15 20
Figure 10. Anti-DNP primary and secondary antibody response for 4 groups of rabbits with various treatments and primed with 3 mg DNP-OVA for 3 consecutive days. Rabbits were either treated with 30 mg ATGG, which had been absorbed with rabbit-Ig-sepharose-4B, on days 6, 7 and 8 (○——○), or 30 mg normal horse gamma globulin on days 6, 7 and 8 (△——△), or were thymectomized 30 days before the first antigen injection (●——●), or were thymectomized as before and treated with ATGG on days 6, 7, and 8 after the first antigen injection (▲——▲). All animals received supplemental immunization with 50 μg each of BGG+KLH in FCA im as free carrier at day 7 and injected with 5 mg DNP-BGG ip as the secondary immunization at day 35. Each curve represents the mean agglutination titer for 4 animals at each day of bleeding.
FREE CARRIER TREATMENT

- 0 → 0  BGG + KLH  RIG-4B ABS.
- △ → △  BGG + KLH  NHIG
- ● → ●  BGG + KLH  ♂ ONLY
- △ → △  BGG + KLH  ♂ + ATGG

FREE CARRIER

DAYS

0  4  7  10  15  21

DNP-OVA TREATMENT

PRIMARY

DANS-BGG

SECONDARY
is negligible or absent when rabbits are immunized with suboptimal
doses of DNP-OVA was also enhanced in the first two groups suggesting
that treatment with ATGG as late as days 6, 7 and 8 after initial
immunization could in some manner enhance the primary response.

Also shown in Figure 10 are the secondary anti-DNP antibody
levels for young rabbits which were thymectomized 30 days before they
received the same immunization schedule as the previous groups.
Antigen-induced suppression was not observed as evident by the signif-
icant secondary anti-DNP responses.

Effect of adult thymectomy plus ATGG treatment on the prim-
ary and secondary anti-DNP immune responses. The intent of this ex-
periment was to investigate the effect, in any, that treatment with
ATGG might have on the anti-DNP response in rabbits thymectomized
30 days before. The animals were immunized with 3 mg of DNP-OVA for
3 consecutive days, as in previous antigenic competition experiments.
ATGG was injected into these animals at days 6, 7 and 8 iv and the
free carrier BGG+KLH were injected sc at day 7. Secondary immuniz-
ation with 5 mg DNP-BGG ip was carried out 35 days after the primary
immunization. Titers (anti-DNP response) were assayed and the results
are shown in Figure 10. The results are unexpected since treatment
with ATGG in thymectomized rabbits diminished the enhanced primary
and secondary anti-DNP response which occurs in animals which are
thymectomized only. The anti-DNP titers were reminiscent to those
seen in animals undergoing antigenic competition. It may be possible
that ATGG treatment of thymectomized rabbits depletes a helper T cell population and therefore memory is severed or limited. This observation, when coupled with the results seen in those experiments where rabbits were treated with 60 mg ATGG and showed diminished primary anti-DNP responses, suggested that ATGG may exert a preferential effect on a population of T cells which exert a regulatory suppressive function. However, in the absence of these cells, depleted by adult thymectomy, ATGG treatment may deplete helper T cells which are necessary to cooperate with macrophages and B cells to mount a T cell-dependent immune responses.
DISCUSSION

Adult Thymectomy in the mouse has been reported to; 1) result in the increase of the generation of cytotoxic T-cells cultured from these animals in vitro (20), 2) enhance antibody responses in vivo to thymus-independent antigens (21), 3) increase helper cell activity in response to TNP-SRBC challenge (18), 4) decrease the inducibility of some forms of tolerance (22), 5) result in the ablation of the thymic suppressor cell response induced by injection of Complete Freunds Adjuvant (23), and 6) deplete Ly $123^+$ cells by 50% in the spleens of thymectomized mice (24). These findings suggest that a short-lived thymus-derived lymphocyte which possess a regulatory suppressor function is depleted from the peripheral tissue which curtails inflated immunological responses under a variety of conditions and in a variety of systems. In the rabbit system, Tada et al (10) have reported that adult thymectomy produced an increase in the primary antibody response to T-dependent antigen and the antibody exhibited an increased avidity in binding antigen.

We report that adult thymectomy in the rabbit performed 30 days prior to experimental immunization will result in enhanced antibody production against the hapen DNP when the animal is immunized with suboptimal doses of antigen (doses that do not provoke appreciable levels of antibody in non-thymectomized animals.) A similar observation was made for rabbits treated with horse anti-rabbit
thymocyte gamma globulin and immunized at the same with suboptimal doses of DNP-OVA. It is thus tempting to hypothesize that thymectomy results in the depletion from peripheral tissue of a short-lived thymus cell with regulatory function or eliminates the source of an inducer which has a peripheral effect on the maturation of regulatory cells. Horse anti-rabbit thymocyte gamma globulin treatment might be acting upon the same regulatory cells after they have peripheralized, and neutralize their suppressive regulatory effect upon being confronted with antigen which results in enhanced responses to sub-optimal antigen doses. Treatments of rabbits with HATGG which were immunized with optimal DNP-OVA doses (10 mg) does not enhance the anti-DNP response, which also suggests that the regulatory function is triggered by lower doses of antigen than are necessary to provoke "helper" function to promote appearance of detectable circulating antibody, since thymectomy or HATGG treatment result in detectable antibody responses to normally suboptimal antigen doses.

Since T-suppressor cells are thought to mediate or regulate the depressed antibody responses observed during antigenic competition, we utilized ATGG in such a system in an attempt to alleviate this phenomenon. Rabbits primed with sub-optimal doses of DNP-OVA would produce a vigorous secondary anti-DNP response 5 weeks later when immunized with DNP-BGG If they had been injected with carrier (BGG) 4 weeks prior to the second immunization. If the rabbits were injected with a competing heterologous carrier, KLH, at the same time
they were given BGG, then the secondary antibody response to DNP upon immunization with DNP-BGG was no better than control primary responses (antigenic competition). When ATGG was administered at the same time that the competing carriers were injected, antigenic competition was eliminated and vigorous secondary anti-DNP antibody response was seen upon immunization with DNP-BGG. Neither normal horse gamma globulin nor ATGG absorbed with thymocytes would alleviate antigenic competition, however ATGG absorbed with rabbit immunoglobulin would. In addition rabbits thymectomized 30 days prior to experimental manipulation did not show antigenic competition. Thus, it again appeared that ATGG treatment and short term adult thymectomy both acted upon a regulatory cell which is operative during antigenic competition.

The HATGG was raised in a horse through immunization with rabbit thymus cells, the great majority of which were probably immature thymocytes. Many investigators have reported that in vivo administration of ALS or ATS into the mouse suppressed the humoral response to subsequent injections of SRBC and other T-dependent antigens (25). In similar studies others have reported treatments of animals with ALS had little effect on the magnitude of the humoral response to T-dependent antigens (26). These varied results probably reflect a variation in the specificity of the anti-sera preparations which may have been provoked by the composition of the lymphoid cell suspensions used to immunize the producer. Bullock et al (27) recently suggested that the splenic Ly 123+ lymphocytes in the mouse is bi-directional in that this cell population can become helper or regulatory in func-
tion. It is premature to propose the existence of such a cell for the rabbit and thus predict or hypothesize the origins of helper and suppressor function for the thymus derived cell in the rabbit.

Fradelizi et al (28) have attempted to identify a cell marker for rabbit T cells and compared the sedimentation properties of marker carrying cells with those of cells carrying immunoglobulin markers. Likewise, Navran and Archer (29) recently reported the isolation of a rabbit T-lymphocyte specific antigen. They found that the purified T cell antigen has a molecular weight of approximately 12,000 and contains 2.5% carbohydrate. However, these studies have not associated the antigen markers to a functionally distinct population of T cells, be it amplifier or regulatory such as have been identified in the mouse system. Since lymphocytic markers for the rabbit are not yet clearly defined, a situation hindered by the lack of inbred rabbits available in reasonably large numbers, it is therefore not possible at this time to identify the population or subpopulation of lymphocyte which is depleted by thymectomy or inactivated/activated by ATGG treatment. We suggest that it would appear that the regulatory cells retain some of the membrane antigens acquired during thymic residence which account for the action of ATGG on regulatory interference; however, a positive action being directed toward a helper cell function or effect on the macrophage or bone marrow derived cell could not be discounted. Currently, attempts to produce antisera against specific markers for studies of regulatory and amplifier T-lymphocytes are in progress in our laboratory.
REFERENCES


Effect of Goat anti-rabbit thymocyte IgG (GATGG) on the primary immune responses to DNP-OVA. In our previous studies, ATGG was produced from horse and we were interested to investigate possibilities that ATGG could also be produced in other animal species. Thus the ability of ATGG that were produced in goat to enhance the primary immune response to sub-optimal dosage (3 mg) of DNP-OVA was investigated. Three groups of rabbits each received the same dosage regimen (15, 30, or 60 mg three times) as that used for HATGG injection and then they were immunized with 3 mg DNP-OVA for 3 consecutive days. The ATGG from goat was standardized with HATGG to the same cytotoxic index before injection to rabbits in order to achieve a meaningful comparison. The results of these experiments were depicted on Figure 11. Under these conditions, optimal dosage of GATGG for maximal enhancement was found to be 30 mg of GATGG. Surprisingly, this dosage corresponds to the dose of HATGG. Thus ATGG could be effectively produced both in horse and goat for the enhancement of the primary immune response to suboptimal doses of DNP-OVA.

Effect of normal thymocyte infusions into thymectomized rabbit on the anti-DNP antibody response. The observation that adult thymectomy in the rabbit enhanced the primary anti-DNP response and would alleviate antigenic competition led us to investigate the notion that infusion of normal thymocytes from littermates into thymectomized
Figure 11. Anti-DNP primary antibody response for rabbits immunized with 3 mg DNP-OVA for 3 consecutive days and treated with Goat anti-thymocyte gamma globulin (GATGG) at a dosage of either 15, 30, or 60 mg for 3 days at one day before, same day, or one day after the first antigen injection. Each curve represents the mean agglutination titer for 4 animals at each day of bleeding.
GATGG DOSE

- 0 0 15 MG
- • • 30 MG
- △ △ 60 MG

GATGG

DAYS
animals could abolish the enhancing effect (repopulation of T suppressor cells). Thymocytes from littermate (9-weeks old) of thymectomized rabbits were harvested, washed and standardized to $2 \times 10^9$ cells/ml. 2.5 ml of this cell suspension was infused into recipient animals which had been thymectomized 30 days before. At this concentration of thymocytes, it was possible to infuse 2 recipients with cells from a single thymus. This dose was selected because a total of 4 recipients were used in this experiment. The recipients after receiving thymus cells were immunized with 3 mg DNP-OVA on the same day of infusion and for 3 consecutive days. Antigenic competition was induced when the rabbits were immunized at day 7 post first antigen injection with 50 ug each of BGG and KLH in FCA and four weeks after this supplemental immunization, the rabbits were immunized with 5 mg DNP-BGG ip. The primary and secondary anti-DNP response for this experiment are shown in Figure 12. Neither the primary or secondary anti-DNP responses were suppressed and if anything, they were slightly enhanced when compared to the thymectomized rabbits alone. There are several possibilities to explain this result. There are reports for the guinea pig system (Armerding, 1975; Katz et al, 1971) that showed, in an in vivo anti-hapten response, that the requirement for carrier recognition can be abrogated by a concomitant graft-versus-host reaction and that the optimum time for hapten challenge in guinea pig was six days after transfer of allogeneic non-specific activation of B lymphocyte function. (allogeneic effect) might have been responsible for the enhanced primary and
Figure 12. Anti-DNP primary and secondary antibody responses for rabbits thymectomized 30 days before first antigen injection (O-----O), or thymectomized 30 days before and infused with $5 \times 10^9$ thymocytes from 9-week old littermates at the same day of first antigen injection (O-----O). Rabbits were primed with 3 mg DNP-OVA at days 0, 1 and 2, supplementally immunized with 50 ug each of BGG+KLH im in FA at day 7 and 4 wks later received 5 mg DNP-BGG ip as the secondary immunization. Each curve represents the mean agglutination titer for 4-8 rabbits at each day of bleeding.
**TREATMENT**

- • • • o • et and THYMOCYTE INFUSION

**PHA TITER**

<table>
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<tr>
<th></th>
<th>0</th>
<th>64</th>
<th>256</th>
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![Graph showing PHA titer over days with treatments](image)

- **DNP-OVA** and **BGG KLH**
- **DNP-BGG**
secondary anti-DNP response even though regulatory cells may be present. Furthermore, in an allogeneic system such as the rabbit, it is possible that the intact regulatory cell could not exert its function across the genetic barrier, however the thymus extract described in the following experiment does exert its effect across the barrier, therefore the possibility of genetic barrier is not attractive. Clearly the datas are inconclusive and we are ignorant of the detailed interactions between infused thymocytes and host lymphoid cells and macrophages, thus experiments of this design were not pursued further in this direction.

The last part of the dissertation research was an attempt to delineate the antigenic specificities of the antibody activity or activities in our ATGG preparation responsible for mediating the enhancing effects observed. There were reports by Tada et al (1975) that soluble suppressor factors elaborated by T cells in the thymus and spleen mediated the primary and secondary responses and it was of interest to investigate if there are factor(s) which suppress the immune response.

**Soluble nonantigen-specific suppressive activity of thymus extract.** In an attempt to investigate the possibility that suppressor T cells in the thymus and spleen elaborate soluble factors in the rabbit as described by Tada and his co-workers (1975) in the mouse system. Soluble extracts were prepared from spleen and thymus of normal and immunized rabbits by the method described by Taniguchi
et al (1974). Donor rabbits were immunized im with 10 mg of OVA in FCA and 14 days later the rabbits received a booster injection of 10 mg OVA sc. Animals were killed 2 weeks after the second injection and the thymus and spleen were removed. Normal rabbits were also killed and their thymus and spleen were used to prepare soluble extracts to serve as controls. The thymus extracts from immunized and normal rabbits were injected into normal recipients which were subsequently immunized with the optimal dose of DNP-OVA (10 mg, 3 X). The anti-DNP response was assayed and is shown in Figure 13. Unexpectedly rabbits injected with thymus or spleen extracts from either hyperimmunized or normal donors showed suppressed anti-DNP responses. These results were confirmed in three separate experiments. Thus it appeared that soluble extracts from both hyperimmunized and normal rabbits were able to, in some way, suppress the anti-DNP primary immune responses. The thymus is known to contain a heterogenous cell population and it was of interest to attempt to identify which cells within the thymic populations are responsible to contributing the suppressive activity present in the soluble extract. Observations from several laboratories indicate that corticosteroids such as hydrocortisone acetate exert a cytotoxic effect on the majority of thymus cells (T-1, immature cells) and that the residual population which is enriched contains mature and immunologically competent lymphocytes (T-2 cells) (Blomgren and Anderson, 1969; 1971). It was decided to investigate whether hydrocortisone acetate treatment of donor rabbits had any effect on the elaboration of soluble suppressive extracts. Normal, unimmunized rabbits were injected ip with
Figure 13. Anti-DNP primary antibody response for rabbits immunized with 10 mg DNP-OVA (optimal dosage) for 3 consecutive days, and either injected with saline alone as control (△—△), or thymus extract from thymus of OVA-primed donor (●—●), or thymus extract from thymus of normal rabbit donor (○—○), or thymus extract from thymus of hydrocortisone acetate-treated rabbit donor (△—△). All thymus extracts were injected one hour before the first antigen injection and with the same route of antigen injection i.e. iv. Each curve represents the mean agglutination titer for 3-5 animals at each day of bleeding.
ANTI-DNP RESPONSE - WHOLE THYMUS EXTRACT

CARRIER IMMUNIZED •
NON IMMUNIZED O
CORTISONE TREATED △
NONE ▲

DAYS

4 7 10 15 21 28 35
a 250 mg of hydrocortisone acetate suspended in saline. The animals were killed 48 hours later and soluble extracts were prepared from the cortisone-resistant cells and injected into recipients that were subsequently immunized with 10 mg of DNP-OVA on the same day (3 consecutive days). Another approach used was to thymectomize rabbits and 4 weeks later, spleen extracts were prepared from these animals and injected into recipients as discussed above. This approach was prompted by the observations in the previous experiments that thymectomized rabbits after 30 days develop enhanced primary anti-DNP response and exhibit an absence of antigenic competition. The result of these experiments are shown in Figure 14.

The suppressive activity was absent in thymus extracts from hydrocortisone treated donors and in spleen extracts from thymectomized rabbit. The anti-DNP response in the recipients was not significantly diminished when compared to control responses.

Next we attempted to remove the suppressive activity in the soluble extracts by adsorbing the extract on various immunoadsorbents prepared on Sepharose 4B beads coupled to the protein carrier OVA, or ATGG or NHGG or 4B beads alone to serve as a control. The suppressive activity in extracts after absorption was tested by injecting the effluents into recipients, with care being taken to correct for dilution effects. The results are shown in Figure 14. The suppressive activity was not absorbed out by the protein carrier OVA or by NHGG or by Sepharose 4B alone. Thus, it appeared that the suppressive activity in the thymus extract did not have an affinity
Figure 14. Anti-DNP primary antibody response for rabbits immunized with 10 mg DNP-OVA for 3 consecutive days and received thymus extracts from thymus of normal rabbit donors or spleen extracts from spleen of rabbits thymectomized 30 days before (○—○). The thymus extract injected into the recipients were either absorbed with OVA-4B (○—○), NHIG-4B (Δ—Δ), or ATGG-4B (▲—▲). All extracts were injected one hour before the first antigen injection and both antigen and extracts were injected iv. Each curve represents the mean agglutination titer for 4 animals at each day of bleeding.
ANTI-DNP RESPONSE - THYMUS EXTRACT PREP.

OVA-4B ABS.  ○
NHIG-4B ABS.  △
ATGG-4B ABS.  ▲
Θ↑ SPLEEN  ●

DAYS

4  7  10  15  21  28  35

256

64

16

4
for the carrier (not carrier-specific), nor for horse immunoglobulins, or for Sépharose 4B. On the other hand, the suppressive activity was absorbed out by the ATGG-sepharose 4B immunoabsorbent since there was not suppressive activity in the eluents which when injected into recipients showed normal anti-DNP responses.

In order to study further the suppressive activity, the immunoadsorbents were reacted with thymus extracts and incubated at 4°C for 4 hours. After the incubation period, the non-specificly bound materials were washed away with 0.01 M PBS, pH 7.6. Next the specifically bound materials were eluted with 3 M NaSCN in PSB. The eluted materials were collected and dialyzed extensively with two changes of PBS and two changes with distilled, deionized water and then lyophilized. The lyophilized materials were resuspended in 1 ml of saline and 0.5 ml of this material was boiled in dissociation buffer at 100°C for two minutes and then cooled in the refrigerator at 4°C. The eluents were then run in SDS-PAGE. The stained pattern of one of these experiments are shown in Figure 15. The HATGG-eluted fraction showed a distinct band with molecular weight which corresponds to approximately 12,000 daltons. This finding is in agreement with Navran and Archer (1978). Further experiments are needed to characterize this band as to chemical composition(s) and biological function.

The interpretation of the results from the soluble suppressor factor studies argues for the existence of non-antigen-specific suppressor T cells that regulate the primary immune response. Whether this suppressor factor operated at the level of induction of
Figure 15. SDS-PAGE pattern of thymus extract eluted with 3 M NaSCN. Lines 1 and 2 are thymus extracts absorbed with Sepharose 4B alone or NHIG-Sepharose 4B respectively. Lines 3–6 are thymus extract absorbed with ATGG-Sepharose 4B. The specific band on lines 3–6 corresponds to a molecular weight of approximately 12,000.
helper T cells or suppressing B cell differentiation and/or function directly is not known yet. Nevertheless, it is clear that ATGG can remove the suppressive activity this factor(s). The experiments in progress include the injection of eluants from 3 M NaSCN into recipients to restore the suppressive effect and the characterization of the eluants.
BIBLIOGRAPHY


VITA

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Major Field: Microbiology

Title of Thesis: Effects of Horse Anti-rabbit Thymocyte Serum and Adult Thymectomy on the Anti-hapten Responses In Vivo

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