1980

Effects of thymectomy on the immune response of Syrian hamsters

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THE EFFECTS OF THYMECTOMY ON THE IMMUNE RESPONSE OF SYRIAN HAMSTERS

By
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B.A., Rutgers College, 1977

Presented in partial fulfillment of the requirements for the degree of
Master of Science
UNIVERSITY OF MONTANA
1980

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Date 3/25/81
The Effects of Thymectomy on the Immune Response of Syrian Hamsters
(93 pp.)

Director: John E. Coe

Following thymectomy in a number of species, severe impairment of both the humoral and cell-mediated immune responses are observed. These immunological impairments are attributed to the absence of thymus-derived lymphocytes (T-cells). The role of the thymus in the development of immunological responsiveness and the function of T-cells in Syrian hamsters have not been extensively studied. T-cell depletion was accomplished through neonatal thymectomy (NTx) and adult thymectomy followed by irradiation and repopulation with bone marrow cells (ATxIR).

Approximately two thirds of the neonatally thymectomized hamsters (Tx-nonresponders) failed to elicit a detectable Ab response after challenge with five µg of hen egg albumin (HEA) in complete Freund's adjuvant (CFA). This abrogated anti-HEA response was evident in both the IgG1 and IgG2 classes of 7S-gamma globulins. The remaining neonatally thymectomized animals (Tx-responders) demonstrated near normal anti-HEA responses. These animals therefore possessed some degree of T-cell immunity suggesting that neonatal thymectomy does not consistently yield T-cell depleted animals. All ATxIR hamsters demonstrated no detectable anti-HEA response. These results indicated that T-cells were necessary for an anti-HEA response in Syrian hamsters.

The allograft response of T-cell depleted animals was determined with skin grafts from the CB inbred strain of hamsters. Approximately 88 percent of neonatally thymectomized hamsters rejected allogeneic skin grafts. These animals included both AB-responder and nonresponder animals suggesting that an allograft response was very difficult to suppress by neonatal thymectomy. ATxIR animals accepted allografts, suggesting a greater degree of T-cell depletion.

The proliferative response of lymph node cells to the T-cell mitogens, ConA and PHA, and the B-cell mitogen, LPS, was measured in a small percentage of thymectomized hamsters. Tx-responder, Tx-nonresponder, and Tx-nonresponder, nonrejector animals demonstrated normal or near normal responses to ConA and PHA. The mitogenic response to LPS was normal in all animals. The mitogen responses of ATxIR hamsters were not determined. These results indicated that ConA- and PHA-reactive cells were present in all thymectomized animals. However, these mitogen reactive cells do not appear to function as helper cells in an Ab response or in the initiation of a DTH response.
ACKNOWLEDGMENTS

With my sincerest gratitude and appreciation, I would like to thank Dr. John E. Coe for his guidance and friendship throughout the course of my studies at the Rocky Mountain Laboratory.

Also, I would like to thank the members of my Master's committee, Drs. G. L. Card, C. A. Speer, and W. E. Hill.

Additional gratitude is extended to Dr. R. N. Ushijima who provided the initial impetus for my research at the Rocky Mountain Laboratory.

Special thanks are given to Dr. Kenneth B. Von Eschen whose guidance and friendship provided a stabilizing force throughout my studies.

Finally, with love I would like to thank my parents, Nicholas and Emma, for their silent and patient sacrifices over the years.

This work was made possible, in part, by the Public Health Service, whom I graciously thank for providing essential funding and the facilities of the Rocky Mountain Laboratory.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>Historical Overview</td>
<td>1</td>
</tr>
<tr>
<td>Immunoglobulin Classes and Helper, Amplifier, and Suppressor T-cells</td>
<td>7</td>
</tr>
<tr>
<td>Cell Surface Markers and Subpopulations of T-cells</td>
<td>11</td>
</tr>
<tr>
<td>Immunoregulatory T-cell Circuits</td>
<td>13</td>
</tr>
<tr>
<td>Hamster Immunology</td>
<td>21</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>Animals</td>
<td>26</td>
</tr>
<tr>
<td>Neonatal Thymectomy</td>
<td>26</td>
</tr>
<tr>
<td>Adult Thymectomy</td>
<td>27</td>
</tr>
<tr>
<td>Preparation of Bone Marrow Cells</td>
<td>28</td>
</tr>
<tr>
<td>Lethal Irradiation of Adult Thymectomized Hamsters and Repopulation with Bone Marrow Cells</td>
<td>28</td>
</tr>
<tr>
<td>Preparation of Hen Egg Albumin for Immunization of Hamsters</td>
<td>28</td>
</tr>
<tr>
<td>Serum Collection from Immunized Animals</td>
<td>29</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Sensitization of Sheep Red Blood Cells with HEA</td>
<td>29</td>
</tr>
<tr>
<td>Determination of Anti-HEA Titers from the Sera of Immunized Hamsters</td>
<td>30</td>
</tr>
<tr>
<td>Determination of Ig Class Specific Anti-HEA Response in Immunized Hamsters: Immunoelectrophoresis and Autoradiography</td>
<td>30</td>
</tr>
<tr>
<td>Immunoelectrophoresis</td>
<td>30</td>
</tr>
<tr>
<td>Autoradiography</td>
<td>31</td>
</tr>
<tr>
<td>Mixed Lymphocyte Reaction</td>
<td>32</td>
</tr>
<tr>
<td>Preparation of Stimulator Cells</td>
<td>32</td>
</tr>
<tr>
<td>Preparation of Responder Cells</td>
<td>32</td>
</tr>
<tr>
<td>Culturing, Pulsing, and Harvesting the Cells</td>
<td>32</td>
</tr>
<tr>
<td>Skin Grafting</td>
<td>33</td>
</tr>
<tr>
<td>Mitogen Assays</td>
<td>35</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>36</td>
</tr>
<tr>
<td>Physical Condition and Survival of Syrian Hamsters Thymectomized and Sham-thymectomized at Birth</td>
<td>36</td>
</tr>
<tr>
<td>Characterization of Mitogenic Responses in the Lymph Node Cells from Syrian Hamsters</td>
<td>42</td>
</tr>
<tr>
<td>Response to ConA</td>
<td>42</td>
</tr>
<tr>
<td>Response to PHA-P</td>
<td>42</td>
</tr>
<tr>
<td>Response to LPS</td>
<td>45</td>
</tr>
<tr>
<td>Characterization of <em>In Vivo</em> and <em>In Vitro</em> Cell-mediated Immune Responses in Normal SD Syrian Hamsters</td>
<td>45</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Survival of Hamsters Thymectomized and Sham-thymectomized at Birth</td>
<td>37</td>
</tr>
<tr>
<td>2.</td>
<td>Skin Graft Response in Normal Syrian Hamsters</td>
<td>51</td>
</tr>
<tr>
<td>3.</td>
<td>Ig Class Specificity of Anti-HEA Responses in Thymectomized and Normal Syrian Hamsters</td>
<td>61</td>
</tr>
<tr>
<td>4.</td>
<td>Allograft Response in Thymectomized, Sham-thymectomized, and ATx1R Syrian Hamsters</td>
<td>62</td>
</tr>
<tr>
<td>5.</td>
<td>Anti-HEA and Allograft Responses in Thymectomized and Normal Syrian Hamsters</td>
<td>64</td>
</tr>
<tr>
<td>6.</td>
<td>Mitogenic Responses in Normal, Thymectomized, and Sham-thymectomized Syrian Hamsters</td>
<td>65</td>
</tr>
<tr>
<td>7.</td>
<td>Classification of Thymectomized Syrian Hamsters</td>
<td>75</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Immunoregulatory T-cell Networks</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of Thymectomy on the Body Weight and Survival of Young Adult Syrian Hamsters</td>
<td>41</td>
</tr>
<tr>
<td>3.</td>
<td>Dose Response of Hamster Lymph Node Cells to Varying Concentrations of ConA</td>
<td>44</td>
</tr>
<tr>
<td>4.</td>
<td>Kinetic Response of Hamster Lymph Node Cells to 1.0 $\mu$g of ConA</td>
<td>44</td>
</tr>
<tr>
<td>5.</td>
<td>Dose Response of Hamster Lymph Node Cells to Varying Concentrations of PHA-P</td>
<td>47</td>
</tr>
<tr>
<td>6.</td>
<td>Kinetic Response of Hamster Lymph Node Cells to 50.0 $\mu$g of PHA-P</td>
<td>47</td>
</tr>
<tr>
<td>7.</td>
<td>Dose Response of Hamster Lymph Node Cells to Varying Concentrations of LPS</td>
<td>49</td>
</tr>
<tr>
<td>8.</td>
<td>Kinetic Response of Hamster Lymph Node Cells to 5.0 $\mu$g of LPS</td>
<td>49</td>
</tr>
<tr>
<td>9.</td>
<td>Mixed Lymphocyte Reaction</td>
<td>54</td>
</tr>
<tr>
<td>10.</td>
<td>Kinetics of the Antibody Response of Thymectomized, Sham-thymectomized, and Normal Hamsters to 5.0 $\mu$g of HEA-CFA</td>
<td>57</td>
</tr>
<tr>
<td>11.</td>
<td>Immunoelectrophoresis Pattern</td>
<td>59</td>
</tr>
<tr>
<td>12.</td>
<td>Immunoelectrophoresis Pattern</td>
<td>59</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

Ab, antibody
Ag, antigen
ALS, antilymphocyte serum
ATxIR, adult thymectomized, irradiated, bone marrow repopulated
B-cell, bursa-equivalent lymphocyte
BDB, bis-diazobenzidine
BSA, bovine serum albumin
C1, complement
CFA, Complete Freund's Adjuvant
ConA, concanavalin A
CPM, counts per minute
DNA, deoxyribonucleic acid
DTH, delayed type hypersensitivity
Fc, c-terminus of Ab heavy chains
FCS, fetal calf serum
GVHR, graft versus host reaction
H-2, histocompatibility locus 2
HEA, hen egg albumin
HGG, human gamma globulin
HSA, human serum albumin
I-A, immune response region, subregion A
Ia, immune response region antigens
Chapter 1

INTRODUCTION

Historical Overview

Observations in the late nineteenth and early twentieth centuries on animals thymectomized during early life revealed that they were relatively unhealthy, did not gain weight properly, appeared physiologically immature, and usually had a shortened life span (73). In the early twentieth century Beard proposed an important role for the thymus in the physiology of the immune system. He suggested that the thymus produced leukocytes that migrate out of the thymus to create "new centres for growth, for increase and useful work for themselves and for the body" (13). This prophetic hypothesis went relatively unnoticed until the late 1950s and early 1960s.

Archer and Pierce demonstrated that removal of the thymus shortly after birth resulted in an impaired antibody response in young rabbits (1). Coincidental studies by Miller showed that thymectomy within 24 hours of birth severely impaired the allograft response in mice (68). In contrast, animals thymectomized five days after birth showed no immunological impairments. The general health of the thymectomized animals was similar to that described in the studies of the late 1800s. The discovery of the immunological significance of the thymus opened a vast area of research that marked the beginnings of
modern cellular immunology.

The thymus, unquestionably one of the most extensively studied organs of the lymphatic system, is intimately associated with and responsible for a complex immunological apparatus. It is the source of T-cells that can function in a variety of immunological reactions. T-cells play a role in the Ab production to certain Ags (3, 12, 19, 57, 62, 74, 95). They are responsible for graft and tumor rejection and they are required for viral and fungal immunity (73).

The thymus is located in the superior mediastinum. In mammals it is a bilobed structure that arises from the third and fourth pairs of pharyngeal pouches (31). Like other lymphoid tissue, the thymus is composed of a reticular cell network and lymphocytes. Unlike the spleen and lymph nodes, however, the thymic epithelial reticular cells arise from the endodermal germ layer, not the mesenchymal layer. Thymic epithelial cells undoubtedly play an important immunological role; they have been implicated in the restoration of immune responsiveness in lethally irradiated animals (83, 108). Furthermore, extracts from thymic epithelial cells were capable of restoring immune functions in thymectomized animals (53) and cultured fragments of thymic epithelium have restored anti-SRBC antibody production, allograft rejection, and mitogen reactivity when grafted into nude mice (53).

The thymus is the first organ during embryogenesis to become populated with lymphocytes. The origin of thymic lymphocytes was a subject of considerable controversy during the early 1960s. The work of Auerbach suggested that the thymic lymphocytes arose de novo from the thymic epithelium (4-6); however, studies utilizing cells with
distinct chromosomal markers revealed that bone marrow cells could gain entry into the thymus (96). Once inside, bone marrow cells were capable of undergoing extensive proliferation in the cortex to yield the post-thymic precursor cell (PTP) (97). The PTP cells possessed T-cell differentiation Ags, mitogen responsiveness, and the capacity to respond in a mixed lymphocyte reaction (MLR). Therefore, under the influence of the thymic microenvironment, bone marrow cells were induced to differentiate into the precursors of functional T-cells.

Experiments utilizing irradiated mice have given credence to the above hypothesis that bone marrow cells could enter the thymus, undergo "thymic processing," and leave the thymus to populate the peripheral lymphoid tissues. Sham-thymectomized, lethally irradiated mice did not regain immune responsiveness unless they were repopulated with bone marrow or fetal liver cells (6, 44, 45, 71). These experiments and marker chromosome studies confirmed the stem cell origin of the thymus-derived lymphocytes. If T-cells arose de novo as Auerbach suggested, repopulation of the irradiated animals with bone marrow cells would not be required for the acquisition of T-cell functions (6, 44, 45, 71).

After the initial observations of impaired antibody responses in the thymectomized animals (2, 19, 26-32, 44, 45, 57, 58, 59, 66, 70, 72), some workers looked at the thymus as being directly responsible for antibody production. However, all attempts to demonstrate Ab production in the thymus itself or in isolated thymocytes were unsuccessful (50, 64).

In 1966 Claman et al. presented evidence to suggest that there
existed a synergistic collaboration between thymus-derived cells and bone marrow-derived cells in the antibody response. Their studies showed that lethally irradiated animals recovered a full capacity to mount an Ab response only when the animals were repopulated with both bone marrow- and thymus-derived cells. Repopulation with one of the cell populations alone did not restore the response (24). Claman suggested that one cell population was responsible for producing the Ab (effector cells), but only with the collaboration of the other cell population called auxiliary cells. Claman further hypothesized that the bone marrow-derived cells were responsible for Ab production and that the thymus-derived cells were the auxiliary cells. This two-celled mechanism of antibody synthesis suggested that there existed a division of labor in the immune response.

This division of labor was elegantly demonstrated in the 1960s via chickens as immunological models. Chickens possess a unique organ, the bursa of Fabricius. The bursa is a lymphoreticular organ located along the intestinal tract near the cloaca. In the late 1950s a graduate student named Bruce Glick performed growth studies on surgically bursectomized chickens. After the completion of the studies, Glick utilized the chickens in an undergraduate teaching lab to demonstrate the production of antibodies against the O Ag of Salmonella typhi. Glick observed that the chickens failed to produce Ab after immunization. Chickens that had not been bursectomized showed normal Ab responses (48). Mueller, Wolfe, and Cote also reported that bursectomized chickens failed to synthesize Abs against bovine serum albumin (BSA) (48).
Aspinall and Meyer, studying the homograft response in chickens, reported that bursectomy had no effect on the homograft response (48); however, chickens that were thymectomized revealed an impaired homograft response. The findings by Glick, Mueller, Aspinall, and Meyer suggested a functional dissociation of immune cells in chickens. Lymphocytes arising from the bursa (B-cells) were responsible for Ab production while the thymus-derived cells (T-cells) were responsible for homograft reactions.

Szenberg and Warner (98) suggested such an immunological dissociation after their studies corroborated the findings of Glick, Mueller, and Aspinall. Cooper suggested that the immunological dissociation into B-cells and T-cells was probably present in other animal systems (30). The chickens, however, were unique due to the separation of these cell populations into two distinct lymphatic organs. The bursa-equivalent in mammals has yet to be identified; the gut-associated lymphoid tissue may be a prime candidate.

Based upon the immunological studies in chickens, a model emerged for the development of immunologically active lymphocytes. Pluripotent stem cells arising from the bone marrow could migrate to the bursa of Fabricius (or its mammalian analog) or the thymus. Upon entry into the bursa, bone marrow cells could proliferate and differentiate into the precursors of Ab-forming cells (B-cells). Extensive mouse studies have revealed that B-cells expressed surface immunoglobulin, Ia antigens, and mouse-specific B-lymphocyte Ag (85). These surface markers were not found on mouse bone marrow cells. This suggests that the markers were expressed during the development of mature
B-cells. Upon appropriate antigenic stimulation, the B-cells experienced proliferation and differentiated into plasma cells that actively synthesized Abs.

The thymus served as the microenvironment for the differentiation of bone marrow cells into thymocytes. The maturation of thymocytes proceeded in distinct stages. The first thymocyte to appear (the immature thymocyte) possessed no demonstrable T-cell functions. It was sensitive to cortisone treatment and it was found in the cortical region of the thymus. These cells expressed the theta, Ly, and TL antigens. The next cell to appear during thymocyte differentiation was the PTP. These cells expressed the theta and Ly antigens, but they no longer expressed the TL antigens. The PTP cells possessed limited immunologic functions. They were found in the medullary regions of the thymus. The PTP cells left the thymus and populated the peripheral lymphoid organs, notably the spleen and lymph nodes.

In the microenvironment of the spleen or lymph nodes and under a hormonal influence by the thymus, the PTP cells matured into fully functional T-cells. These functional T-cells expressed theta and Ly antigens and demonstrated a wide variety of immune functions. The functional T-cells could participate in Ab production, graft rejection, DTH, MLR, GVHR, and cell-mediated cytotoxicity. Peripheral T-cells were fully capable of restoring immunological responsiveness in thymectomized animals (46, 96, 97) whereas the thymocytes could not.

Following the original reports of Archer, Pierce, and Miller (1, 68, 70), numerous other studies corroborated these early findings. The later works revealed that thymectomy resulted in an impaired
humoral response to the following Ags: SBRCs (12, 42, 74, 80), BSA (12, 19, 57, 80), HEA (3), TMV (95) and KLH (62). Antigens of this nature were called thymic-dependent Ags because the specific Ab response by an animal was abrogated following thymectomy.

Immunoglobulin Classes and Helper, Amplifier, and Suppressor T-cells

Thymectomy not only resulted in an impairment of a specific antibody response, it also appeared to affect the relative serum concentrations of certain Ig classes. Thymectomized rats showed decreased levels of IgG1 (2) and thymectomized mice demonstrated low serum levels of IgG1 and IgG2 (39). Congenitally athymic nude mice showed drastically reduced levels of IgG and IgA (11, 63) with the reduction of IgG1 being particularly severe.

Taylor and Wortis reported that in thymectomized mice immunized against SRBCs, the IgG1 response was the most severely affected (102). Torrigiani reported that in adult thymectomized ALS treated mice (a treatment that mimicked the T-cell depletion induced by neonatal thymectomy), very low levels of antibody were produced in response to HSA and KLH. Radioimmune precipitation with class specific antisera revealed that the reduction in the overall antibody response was due primarily to a reduction in the IgG1 class (104). The IgG1 response therefore appeared to be more dependent on T-cell help than the other Ig classes.

The aforementioned studies in T-cell depleted animals (11, 104) and nude mice (11, 63) demonstrated that different Ig classes showed varying degrees of T-dependence. These observations suggested
that specific regulatory mechanisms existed between T- and B-cells in the generation of an Ab response. The primary cellular requirements in an Ab response was the presence of Ag-reactive B-cells of a given Ig class and specific helper T-cells. These helper cells possessed Ag specificity as well as the specificity for helping B-cells committed to a particular Ig class.

This concept was well-illustrated in the allotype suppression studies reported by the Herzenbergs (52). Allotype suppression was induced by injecting neonatal mice with antiserum specific for a corresponding mouse Ig allotype; in this study the allotype was referred to as Ig-lb. Treatment with allotype-specific antisera prevented the development of the helper T-cells required for Ig-lb synthesis. As adults, the mice demonstrated drastically reduced serum levels of Ig-lb whereas no other Ig classes were affected. When the allotype-suppressed animals were challenged with a T-dependent Ag, they failed to make an Ig-lb specific response. T-cells isolated from these animals were not capable of providing help to hapten-primed Ig-lb memory B-cells. They could, however, provide help to B-cells expressing different Ig-class specificity (52).

The Herzenberg studies suggested that allotype suppression specifically removed a particular subpopulation of helper T-cells involved in Ig-lb synthesis. These findings indicated that distinct subpopulations of T-cells were responsible for the induction of a particular Ig class. Removal of these subpopulations by allotype suppression, thymectomy, or ALS treatment could result in an impaired synthesis of the Ig class involved.
In contrast to IgG₁ (which was especially dependent on T-cell help), IgM did not require T-cells for the induction of Ab synthesis. The first Ig class to appear during a primary Ab response was IgM. This response was rather short-lived; it was, for the most part, T-independent. T-independent Ab responses did not require specific T-cell help. They were generally not affected after thymectomy. Antigens that elicited such responses were generally long polymeric substances composed of repeating units. Classic T-independent Ags were pneumococcal polysaccharide (SSS-III), polyvinylpyrolidone (PVP), and lipopolysaccharide Ags from the enteric bacteria. T-independent responses to SSS-III differed from T-dependent responses in two ways: (1) immunization with SSS-III resulted in strictly an IgM-specific response (8) and (2) secondary challenge with SSS-III did not result in a heightened IgM response and detectable IgG specific Abs were not observed (8). (Secondary responses to classical T-dependent Ags were usually characterized by a predominantly IgG response.)

The role of T-cells in the regulation of the anti-SSS-III response was extensively studied by Baker (7, 10). Treatment of mice with antilymphocyte serum (ALS) resulted in a significant increase in the anti-SSS-III response. Baker suggested the presence of a suppressor cell that acted to regulate the anti-SSS-III response. Removal of this suppressor cell by ALS alleviated the suppression; this resulted in an enhanced Ab response. When syngeneic thymocytes were injected into the ALS-treated animals, suppression was restored and the antibody titers returned to normal levels. Thus it appeared that the ALS-sensitive cell was a suppressor T-cell.
Further studies by Baker and Prescott, concerning the cellular basis of the anti-SSS-III response, revealed the presence of two distinct subpopulations of T-cells involved in the regulation of the response. The two subpopulations were referred to as amplifier T-cells ($T_A$ cells) and suppressor T-cells ($T_S$ cells). The data suggested that $T_A$ and $T_S$ cells interacted to maintain a regulatory balance in the anti-SSS-III response (10).

$T_A$ cells did not function as helper cells. They were not required for the initiation of the anti-SSS-III response. Because B-cell proliferation occurred after antigen stimulation, the $T_A$ cells drove the activated B-cells into further proliferative activity. This resulted in a net increase in the number of B-cells responding to Ag and, thus, amplified the B-cell response.

The $T_S$ cells acted to suppress the $T_A$ and the B-cell responses. $T_S$ cells could suppress the amplification of B-cells by interacting with the $T_A$ cells or the B-cells directly. Treatment with ALS preferentially removed the $T_S$ cells, shifting the regulatory balance in favor of the $T_A$ cells and the B-cells. These cells could now proliferate freely. This resulted in an enhanced Ab response. Regulation of the SSS-III response required the interaction of two distinct T-cell subsets with opposing functions: amplification and suppression. The interactions of $T_A$ and $T_S$ cells provided a homeostatic mechanism for the regulation of a B-cell Ab response.
Cell Surface Markers and Subpopulations of T-cells

The discovery of unique cell markers on lymphocytes has been proven to be a powerful tool in elucidating the cellular nature of immune responses. The presence of surface Ig on B-cells and the theta Ag of T-cells has served as a primary means of identifying B-cells and T-cells.

Subpopulations of T-cells were identified in mice by Ly alloantigens. The Ly 1 and Ly 23 alloantigens were expressed exclusively on T-cells (60). They appeared to be stable genetic markers associated with functionally distinct sets of T-cells. Kisielow et al. (60) demonstrated that the treatment of T-cells with anti-Ly 1 serum plus complement (C^) abolished the helper function of the T-cells. Treatment of T-cells with anti-Ly 2 and C^ resulted in a marked decrease in cell-mediated cytotoxicity; however, the helper activity was not affected. Ab helper T-cells therefore preferentially expressed the Ly 1 antigen with the cytotoxic T-cells expressed the Ly 23 antigen.

Cantor and Boyse (20) demonstrated that the maturation of thymocytes to yield T-cells expressing distinct Ly phenotypes occurred in the absence of Ag. Based on the expression of Ly phenotypes, three sets of T-cells were identified: Ly 1^ cells, Ly 23^ cells, and Ly 123^ cells. Cantor and Boyse demonstrated that the Ly 123^ cells were the first to appear during development. They were susceptible to the effects of adult thymectomy. Ly 123^ cells may have corresponded to the PTP cells (97). Ly 1^ and Ly 23^ presumably arose from Ly 123^ cells and were relatively resistant to the early effects of adult
thymectomy (20).

Cantor and Boyse demonstrated that T-cells expressing the Ly 1^ phenotype were capable of generating help in vitro. Ly 23^ T-cells possessed suppressor and cytotoxic activity (20, 21). Their studies also showed that two subsets of T-cells could collaborate in the generation of a cytotoxic response. The development of Ly 23^ cytotoxic T-cells was amplified by Ly 1^ cells (21). During the generation of a cytotoxic response, the Ly 1^ cells recognized a different Ag than that recognized by the Ly 23^ cells. Ly 1^ cells recognized an I region difference while Ly 23^ cells recognized K and/or D region differences.

Further characterization of the Ly 1^ helper cells and the Ly 1^ amplifier cells was achieved with the use of Ia antiserum. These studies demonstrated that the Ly 1^ helper cells expressed Ia antigens on their surface while the Ly 1^ amplifier cells did not. The presence of Ia antigen may have represented a functional cell marker for a different differentiative pathway (105). Suppressor T-cells were shown to express the phenotype Ly 23^ Ia^+. Spleen cells isolated from animals made tolerant to HGG were treated with a variety of antiserums and their suppressor activity was assessed. Only antisera directed against Ly 23^ antigens and Ia antigens abolished the suppressor activity (105).

Studies by Huber demonstrated that the Ly phenotypes represented a stable functional marker in vivo. ATxIR mice (B-mice) were repopulated with Ly 1^ or Ly 23^ T-cells and subsequently tested for immunological capabilities. The B-Ly 1 mice were capable of generating
primary and memory helper functions in an anti-SRBC response. These animals did not demonstrate any cytotoxic activity (54). The B-Ly 23 mice were incapable of eliciting an anti-SRBC response, but they were capable of generating primary and memory killer functions (54). Of interest was the observation that the degree of cytotoxicity was less than that seen in fully reconstituted B-T mice. The absence of Ly 1+ amplifier cells may have been responsible for the lower levels of cytotoxic T-cells. Thus Ly 23+ cells by themselves were capable of generating a cytotoxic response. Optimal cytotoxic responses, however, required an interaction between an Ly 1+ amplifier and an Ly 23+ cytotoxic T-cell (54).

**Immunoregulatory T-cell Circuits**

Richard Gershon and a number of coworkers have proposed that T-cell subsets could interact in an immunoregulatory circuit to regulate a specific Ab response. The studies of Gershon et al. (37, 38, 54) have relied extensively on the use of Ly antisera to obtain relatively purified subpopulations of T-cells.

Highly purified Ly 1+ T-cells were primed *in vitro* with SRBCs. The degree of priming was previously shown to induce optimal suppression when unselected T-cells were stimulated in this manner. When the antigen-primed Ly 1+ cells were added to purified B-cells, a significant antibody response was observed although significant suppressive effects were observed when antigen-primed Ly 1+ cells were added to mixtures of unselected T-cells and B-cells. Therefore, although by themselves Ly 1+ cells were capable of directly inducing Ab synthesis
in B-cells, these Ly$^+$ cells were also capable of inducing a resting set of T-cells to exert profound suppressive effects on the antibody response (37).

Additional studies revealed that the resting T-cell population that was induced by the Ly$^+$ cells expressed the Ly$^{123^+}$ phenotype. The Ly$^{123^+}$ cells were induced to exert potent feedback inhibition of the Ab response. The Ly$^+$ mediated control of the Ab response represented a unique interaction among T-cell subsets. Upon antigen stimulation, Ly$^+$ T-cells could induce B-cells to produce Ab. After a certain degree of help was provided, the Ly$^+$ subset activated a resting set of Ly$^{123^+}$ cells to express potent feedback suppression (54, 105).

Support for the circuit theory came from in vivo studies by Cantor et al. who showed that the same T-T interactions required for the regulation of an in vitro Ab response were also necessary for the regulation of an in vivo Ab response (23, 54). Antigen-stimulated Ly$^+$ cells were capable of generating a significant helper response in B mice. When, however, Ly$^+$ cells were added to unselected T-cell populations and injected into B mice, significant suppression of the Ab response was observed (23, 54). Removal of the Ly$^{123^+}$ cells from the unselected T-cell population prevented the development of suppressor T-cells.

Additional proof came from work done on NZB mice, a strain that spontaneously developed autoimmune diseases. The development of autoimmunity may have been due to the absence of the feedback suppression provided for by Ly$^{123^+}$ cells (23). Experiments with these mice
demonstrated a very low level of Ly 123^ cells. This apparent defect in the Ly 123^ cells prevented sufficient feedback control mediated by the Ly 1^ cells. The loss of this suppression resulted in a relatively uncontrolled Ab response. This defect was believed to be responsible for the development of autoreactive Abs. The equilibrium of this circuit has been perturbed by the Ly 123^ defect. This perturbation resulted in an uncontrolled proliferation of Ly 1^ cells that resulted in an enhanced Ab response. In this system, however, the unsuppressed proliferation of B-cells may have given rise to autoreactive clones of Ab-forming cells.

McDougal et al. (76) corroborated the findings of Gershon. They utilized an *in vitro* thymocyte-macrophage culture system to generate helper T-cells. They found that Ly 1^ cells could supply direct help to B-cells and induce a resting set of T-Cells to exert potent feedback suppression.

McDougal and Gordon (75) have characterized a soluble factor that can replace Ly 1^ cells in an antibody response. The factor was obtained from the supernatants of the *in vitro*-generated helper cells. Immunochemical analyses of this factor revealed it to be a glycoprotein with antigen-binding capacity. It did not react with antisera directed against Ig determinants; it was precipitable with alloantisera. Because the factor could replace helper T-cells, it was presumably secreted by antigen-stimulated Ly 1^ cells. Thus B-cells received the helper T-cell signal via a soluble messenger.

A number of other factors have been isolated from T-cells or culture supernates and characterized. These factors demonstrated
helper and suppressor activities (32, 99, 100, 107, 108). Watson et al. (106) have purified a helper T-cell replacing factor that was obtained from the supernatants of ConA-activated spleen cells. This factor was a protein with a molecular weight of approximately 30,000-40,000 daltons. The factor supplied a direct signal to B-cells that was antigen-specific.

Taniguchi and coworkers (99, 100) have isolated and characterized a T-cell factor that exerted suppressive effects on anti-KLH responses. This was extractable from thymocytes of mice that had been immunized with high doses of the carrier protein. The factor was shown to have Ag-binding capability. It had a molecular weight of 35,000-55,000 and it did not react with anti-Ig Abs. These studies revealed that the suppressive factor functioned at the T-cell level in suppressing helper T-cells.

Additional studies by Taniguchi et al. (100) have shown the T-cell suppressive factor to be a product of a gene mapping within the I region of the mouse H-2 complex. The subregion coding for this molecule was referred to as the I-J subregion. This subregion mapped to an immune response gene. The factor characterized by Taniguchi showed a considerable degree of similarity with the soluble factor characterized by Herzenberg et al. (78). Herzenberg was able to demonstrate that suppressor T-cells expressed a gene that mapped to the I-J subregion and also secreted a suppressive factor that expressed I-J antigens. The similarities between Taniguchi's and Herzenberg's work suggested that suppressor T-cells might preferentially express I-J subregion genes that appeared to play a direct role in the T-cell mediated suppression
Ia antigens were glycoproteins serologically detectable on the surfaces of T-cells, B-cells, and macrophages. These antigens were controlled by genes in the I (immune response) region of the major histocompatibility complex (H-2) of mice (61). The I region was divided into five subregions as defined by distinct recombination events: A, B, J, E, and C. The Ia antigens (I-A and I-J) have been found on helper T-cells and suppressor T-cells, respectively. A number of T-dependent immune responses were controlled by genes within the I region and Ia antigens appeared to play a direct role in regulating these responses.

The presence of Ia antigens on T-cells, B-cells, and macrophages might play an important role in immune regulation. A number of T-dependent immune responses were controlled by genes in the I region of the H-2 complex (88). Ia antigens might serve as messenger molecules from one T-cell to another, or from a T-cell to a B-cell. This message could be conveyed through direct cell contact involving the Ia antigens or through the action of Ia-like soluble factors.

Studies utilizing responder and nonresponder strains of mice revealed that the cellular defect in the nonresponder animals was associated with a lack of Ia antigens on the B-cells of the nonresponder animals. Nonresponder T-cells were capable of responding to the Ag and releasing Ag specific soluble factors; however, the nonresponder B-cells were refractory to the helper effects of the soluble factors (88, 101).

Contrary to the T-cells involved in the regulation of an antibody response, the T-cells involved in the generation of delayed hypersensitivity and cytotoxicity did not appear to express surface Ia
antigens (105). Although these cells have been shown to recognize I region differences (21), Ia antigens did not seem to be expressed on these T-cells. These findings implied that the presence of Ia antigens on T-cells might represent a functional separation of T-cell subsets. Ia+ T-cells appeared to be involved in regulating Ab responses while Ia− T-cells were involved in cell-mediated responses.

Figure 1 represents a proposed interaction of T-cell subsets in the regulation of an antibody response. The initiating events in the circuit might be the stimulation of Ly 1+ cells by the appropriate Ag. Upon stimulation, the Ly 1+ cells would begin to supply direct help to B-cells—most likely through a soluble factor. Upon receiving a signal from both the Ag and the Ly 1+ T-cell, the B-cells would begin to proliferate and mature into Ab-secreting plasma cells. Simultaneously the Ly 1+ I-A+ T-cells would provide signals to both the Ly 123+ I-A+ cells and the Ly 123+ I-J+ cells. Stimulation of the Ly 123+ I-A+ cells would result in the additional generation of Ly 1+ I-A+ helper cells. As this process continued, a considerable degree of help would be supplied to the B-cells.

After a certain amount of Ab has been produced, feedback mechanisms would begin to suppress the antibody response. Ly 1+ cells were capable of inducing the maturation of Ly 23+ I-J suppressor cells from Ly 123+ I-J precursor cells. The appearance of Ly 23+ I-J cells would result in the suppression of the Ly 1+ mediated response. Another mechanism of suppression illustrated in this diagram is the feedback inhibition induced by soluble immune complexes. These immune
Figure 1. Immunoregulatory T-cell Networks

A proposed representation of the regulatory interactions among different T-cell subsets controlling an antibody response.
Thymic seeding

Thymus

T-amplifier cell (helper)

Ly^{123+} I-A^{+} Thy-1^{+}

T-amplifier cell (suppressor)

Ly^{123+} I-J^{+} Thy-1^{+}

Thy-1

F_c R^{+}

Ab + Ag → Ag:Ab complex

synthesis and secretion

T-suppressor cell

T-helper cell

Ly^{1+} I-A^{+} Thy-1^{+}

Ia^{+}

sIg^{+} Ia^{+} FcR^{+}

B-cell

positive effect via soluble factor and/or cell contact

negative effect via soluble factor and/or cell contact

proliferation and maturation

negative effect mediated by soluble immune complex
complexes could bind to the Ly 23\(^+\) I-J cells and possibly enhance their suppressive activity. Thus the production of excess Ab or an excess amount of Ly 1\(^+\) mediated help could result in the feedback suppression of an Ab response.

The interaction of T-cells in an immunoregulatory network provided a fine control mechanism for the regulation of Ab production. These circuits provided a homeostatic mechanism that allowed for the precise regulation of an antibody response. Any malfunction in this network could conceivably result in the perturbation of a normal Ab response, possibly resulting in immune tolerance or autoimmunity.

**Hamster Immunology**

The Syrian hamster was first described in 1839 near Aleppo, Syria. In 1930 a breeding nucleus of hamsters was established. Adler soon discovered their unusual susceptibility to many infectious agents. Adler successfully established models for Kala azar, tuberculosis, brucellosis, and a number of viral and bacterial diseases. Hamsters were shown to be good hosts for grafted and spontaneous tumors (17).

Hamsters proved to be excellent hosts for many viral diseases: lymphocitic choriomeningitis, mumps, measles, rubella, and influenza viruses (103). Eddy demonstrated that polyoma viruses and DNA viruses such as SV40 were capable of inducing tumors in hamsters. Of interest were the two human viruses, adenovirus type 12 and herpes simplex type 2, that were also oncogenic in hamsters.

The unique susceptibility of hamsters to viral infection and oncogenesis would surely warrant an in-depth investigation of their
immune system. For reasons unknown, however, relatively few studies of the hamsters' immune system emerged in the 1950s.

In the late 1950s Hubert Billingham and Bill Hildemann were studying the genetics of the homograft response in hamsters. Up until this time hamsters had only demonstrated very weak homograft responses. This phenomenon was attributed to two factors: (1) Syrian hamsters, severely restricted geographically, showed little polymorphism at their histocompatibility loci and (2) all of the domestically bred hamsters were derived from three original littermates captured near Aleppo. The apparent lack of polymorphism in the histocompatibility genes of hamsters was contrary to the situation found in mice (61).

Billingham and Hildemann demonstrated that, by using proper strain combinations, hamsters were capable of eliciting strong graft rejection. Their data suggested the existence of a few strong but at least several weak histocompatibility genes (15). Billingham et al. (16) later demonstrated the existence of only three histocompatibility loci in hamsters, one being a major locus.

A series of studies by Duncan and Streilein (33-35) revealed the presence of a histocompatibility locus in hamsters. The hamster locus coded for genes controlling graft rejection, MLF, and GVHR. These tests were all T-dependent functions. The presence of a histocompatibility complex in hamsters was significant because genes controlling immune responsiveness were probably located within this region similar to Ir genes in the H-2 locus of mice (61). Too, the H-2 locus in mice controlled helper and suppressor T-cell activity, cell-mediated cytotoxicity, and viral and tumor immunity (61).
Additional studies by Duncan and Streilein (34) demonstrated that the genes controlling graft rejection, MLR, and GVHR were closely linked. This linkage led to the hypothesis of hamsters' major histocompatibility locus. The immune response to BSA was shown to be under single gene control although unlinked to the histocompatibility complex.

In the early 1960s hamster studies began to focus on the role of the thymus and the characterization of hamster Igs. Sherman et al. (89) reported the occurrence of a severe wasting syndrome following thymectomy in hamsters. They found that thymectomy as late as four weeks after birth could result in the wasting disease. Thymectomy was also found to impair the humoral and cell-mediated immune responses (90, 91). The reported late effects of thymectomy were quite remarkable considering the critical time period seen in other animal systems. Mice and rats must be thymectomized before 24 hours after birth in order to observe significant immune impairments (57-59, 68).

Experiments by Roosa et al. (87) contradicted Sherman's findings. Their studies showed that thymectomy within 24 hours of birth resulted in but a slight impairment of the homograft response. Hamsters thymectomized seven days after birth rejected skin grafts as did normal animals. Roosa did not observe the sex-limited wasting syndrome that Sherman described. It was apparent from this study that very early thymectomy was necessary to elicit an immunologic impairment. This finding was in direct contrast to Sherman's work.

Gasser and Loeffler showed that neonatally thymectomized hamsters were more susceptible than normal animals to tumor induction by adenovirus
type 12 (43). Wasting disease was not observed in this study.

In the late 1960s Coe began a systematic characterization of the Ig classes in hamsters. IgG₁ and IgG₂ were identified as 7S gamma globulins (24). Additional studies revealed that only the IgG₂ class was capable of fixing complement (28). A 20S gamma globulin was identified as IgM (26). IgM was dissociable into 7S subunits upon reduction and alkylation. IgM, IgG₁, and IgG₂ all appeared after immunization with a protein Ag; however, the IgM response was only detectable for a short time (26).

Coe also described a unique response to HEA in Syrian hamsters. When hamsters were challenged with HEA in CFA they elicited an IgG₁ and IgG₂ specific response. Immunization of hamsters with HEA in saline resulted in an IgG₁ response with no production of IgG₂. If the HEA saline hamsters were now challenged with HEA in CFA they elicited an IgG₁ response. Immunization of hamsters with HEA in saline resulted in the selective induction of IgG₁ synthesis and the concomitant induction of specific HEA tolerance in the IgG₂ response (26, 27). This phenomena was referred to as split tolerance.

The cellular basis for the split tolerance phenomenon was not completely understood. The complexity of this response and the nature of the Ag would indicate that T-cells might be involved in regulating the anti-HEA response. The anti-HEA response in thymectomized hamsters has not been studied.

The discovery of a major histocompatability complex and the presence of immune response genes in hamsters was evidence that they possessed a complex immune system. Presumably the immune response
would be subject to T-cell regulation. Although T-cell subsets in hamsters have not been identified, their presence in mice would suggest that such a T-cell system should also exist in hamsters.

Hamster lymphocytes were similar to those of mouse and man in that hamster lymphocytes responded to T-cell specific mitogens (ConA and PHA) and the B-cell mitogen (LPS). ConA and PHA were good mitogens for hamster thymocytes, but they did not induce mitosis in hamster bone marrow cells. LPS induced proliferative responses in bone marrow cells, but they did not in thymocytes (51). In addition, Blasecki and Houston (18) have partially purified and characterized hamster T-cells through the use of nylon wool columns and a T-cell specific antisera. This antisera was prepared by immunizing rabbits with hamster brain tissue in much the same way that antitheta antisera is prepared in mice. The existence of a T-cell specific antigen on nervous tissue in hamsters was analogous to the theta Ag system in mice (84).

The aforementioned studies suggested that a T-cell system, much like that found in mice, was present in Syrian hamsters. Theoretically, hamster T-cells participated in regulating Ab responses, graft rejection, tumor rejection, MLRs, and GVHRs. Hamster T-cells expressed unique surface markers that might allow for their identification and characterization. Hamster T-cells were also known to respond to T-cell mitogens. The effect of T-cell depletion has not been thoroughly evaluated in reference to Ig classes of Ab affected, MLR and graft rejection impairment. The present study was initiated to further elucidate the role of the thymus in the immune system of the Syrian hamster.
Chapter 2

MATERIALS AND METHODS

Animals

Outbred male and female Sprague-Dawley (SD) Syrian hamsters were bed and maintained at the Rocky Mountain Laboratory (RML). Inbred CB hamsters were purchased from the Charles River Laboratory, Lakeview, New Jersey. Lewis rats were maintained at the RML.

Neonatal Thymectomy

Newborn hamsters, 2-16 hours of age, were rendered hypothermic by incubation for 8-10 minutes at $-20^\circ$ C in preparation for surgery. The newborn animals were securely fastened to an inverted petri plate that was placed on ice. The ice bath served to maintain hypothermic conditions throughout surgery. Using sterile surgical scissors, a 0.5 cm incision was made through the skin along the sternal line, thereby exposing the rib cage and sternum. Starting just above the xyphoid process, the sternum was split to just below the neck. Using a pair of forceps, the split sternum was gently separated to expose the superior mediastinum. The thymus was visualized in the uppermost part of the superior mediastinum, lying above the heart. Utilizing a Pasteur pipette connected to a gentle vacuum, the thymic lobes were carefully removed without damage to the surrounding tissues. A 5-0 Dermalon
suture (Davis and Geck, American Cyanamid, Pearl River, New York) was used to close the sternal and skin incisions. The animals were maintained at room temperature after surgery or occasionally placed under a heat lamp until consciousness was restored. Fully conscious animals were returned to their litters and observed daily until weaning.

Sham thymectomy employed the identical procedure outlined above except that the thymus was left intact.

**Adult Thymectomy**

Three- to four-week-old male and female Syrian hamsters were anesthetized with 0.1-0.15 ml of a sodium pentobarbitol solution (Nembutal, Abbot Laboratories, North Chicago, Illinois). The animals' upper and lower thoraxes were shaved and cleansed with a disinfectant soap (Phisohex, Winthrop Laboratories, Sterling Drug, Inc., New York, New York). An incision was made through the skin to expose the entire sternum and rib cage. Starting at either the third or fourth rib above the xyphoid process, a small incision was made along the sternum exposing the superior mediastinum. While separating the rib cage with one pair of forceps, the thymus was teased out of the chest cavity with a second pair of forceps. The separated rib cage was then immediately drawn together with three or four sutures so as to diminish the effects of pneumothorax. The skin was closed via surgical staples (Clay Adams, Becton-Dickinson Company, Parsippany, New Jersey).
Preparation of Bone Marrow Cells

Bone marrow cells were obtained from adult female SD hamsters. The animals were anesthetized with ether, exsanguinated, and the femurs and humerus bones removed. The ends of the bones were cut off with bone scissors and, using a syringe, a solution of sterile PBBS (see Appendix A, p. 82) was washed through the bones to flush out the marrow cells. Large aggregates of bone marrow cells were dispersed by repeated aspirations into a sterile Pasteur pipette; the cells were washed three times, counted, and the final concentration was adjusted to $1 \times 10^8$ cells per ml. The above procedure was performed at $4^\circ$ C and the cells were kept at this temperature for the remainder of the procedure.

Lethal Irradiation of Adult Thymectomized Hamsters and Repopulation with Bone Marrow Cells

Thirty to 60 days after surgery, adult thymectomized SD hamsters were lethally irradiated via a General Electric Maximar X-ray unit. The target distance was 24 inches using 250 V, 15 mA, with a 1 mm Cu and a 1 mm Al filter. The rate of exposure was 36 rads/min. with the animals receiving a total of 1,000 rads. All animals received $50 \times 10^6$ bone marrow cells injected intravenously within six hours of irradiation.

Preparation of Hen Egg Albumin for Immunization of Hamsters

Hen egg albumin (HEA), five times recrystallized, was purchased from the K and K Laboratories, Jamaica, New York. A standardized stock
solution of HEA was prepared in a PBS buffer and stored at 4°C for further use. A solution of HEA containing 20 μg/ml was prepared and emulsified with an equal volume of Complete Freund's Adjuvant (CFA) (Difco Labs, Detroit, Michigan). Experimental hamsters were immunized with 0.5 ml of the HEA-CFA emulsion. The injection was evenly distributed among the four footpads.

Serum Collection from Immunized Animals

Sera from immunized animals was collected by retro-orbital sinus puncture using heparinized capillary tubes (Scientific Products, McGaw Park, Illinois). Two hundred fifty ul of blood was obtained and the capillary tubes were stored overnight at 4°C. Plasma was obtained by centrifuging the capillary tubes at 2,000 RPM for 30 minutes. Plasma was stored at -20°C for further use.

Sensitization of Sheep Red Blood Cells with HEA

SRBCs in Alsever's solution were obtained from sheep maintained at the RML. The cells were washed three times with PBS and diluted to yield a packed cell volume of 0.25 ml. The cells were suspended in 18 ml of PBS containing 10 mg of HEA and mixed gently for one minute. Then 1.25 ml of a 1:15 BDB buffer A solution was added to the cells and the suspension was incubated at room temperature for 10 minutes. The suspension was centrifuged at 1,500 RPM for five minutes. The supernatant was removed immediately and the cell pellet was washed two times with PBS. The final washed pellet was resuspended in
50 ml of PBS 0.1 percent BSA buffer to yield a 0.5 percent suspension of HEA-conjugated SRBCs. Efficacy of the sensitization procedure was tested using a standard anti-HEA sera prepared from hyperimmunization of hamsters with HEA-CFA.

Determination of Anti-HEA Titers from the Sera of Immunized Hamsters

Hemagglutination using HEA-sensitized SRBCs was performed in microtiter plates (Flow Labs, Inglewood, California). Twenty-five microliters of immune sera were serially diluted twofold with PBS 0.1 percent BSA buffer. Twenty-five microliters of a 0.5 percent HEA-SRBC suspension was added to each well and the plates were incubated overnight at 4° C. Controls consisted of normal hamster serum, hyperimmune hamster serum, and unconjugated SRBCs. The hemagglutination titer was recorded as the reciprocal \(\log_2\) of the highest dilution showing a positive hemagglutination pattern. Hyperimmune hamster serum consistently demonstrated reproducible hemagglutination patterns.

Determination of Ig Class Specific Anti-HEA Response in Immunized Hamsters: Immunelectrophoresis and Autoradiography

Immunelectrophoresis

Immunelectrophoresis of immune sera was carried out on 1 x 3 inch glass slides. The slides were washed with acid alcohol, dried, and a thin coat of 2 percent agar was spread across each one. The slides were then baked in a drying oven for 20 minutes. Two and one half ml of 2 percent Ionagar #2 (Colob Laboratores, Chicago Heights,
Illinois) in an IEP barbital buffer was layered onto each slide. This was allowed to solidify. A center trough and two adjacent wells were cut into the agar. The wells were removed and approximately five microliters of immune sera were added to each well. The immune sera was electrophoresed against the IEP barbital buffer at 45 V for 90 minutes. The IEP apparatus was kept cool throughout the procedure so as to prevent overheating.

After completion of electrophoresis, the center trough was removed and subsequently filled with rabbit antiwhole hamster sera. The IEP slides were placed in a humidified container and allowed to develop at 4°C for 48 hours.

**Autoradiography**

Developed IEP slides were washed for 72 hours with several changes of PBS. After washing, $^{125}$I-HEA was added to the trough and allowed to diffuse into a gel. After incubation at 4°C for 48 hours, the slides were washed for 72 hours with several changes of PBS. Slides were dried by applying filter paper directly to the gel and then stained with a 0.05 percent Azocarmine G acetate buffer for 20 minutes. Slides were washed twice in a 1 percent glycerin-2 percent glacial acetic acid solution, allowed to air dry, and were placed face down on Kodak Royal Pan film (ASA400) for one week. Slides were removed and the film was developed at 72°F for three minutes. The resultant autoradiographic patterns were correlated directly with the original IEP slides.
Mixed Lymphocyte Reaction

Preparation of Stimulator Cells

Peripheral lymph nodes were removed from outbred SD hamsters and inbred CB hamsters and placed in cold PBBS. Nodes were minced with two sterile blades and expressed through a stainless steel mesh screen. This crude suspension was transferred to a sterile test tube and large clumps of tissue were allowed to settle out. After settling, the upper 80 percent of the suspension was removed with a sterile Pasteur pipette, transferred to a large centrifuge tube, and brought to a total volume of 50 ml with PBBS. Cells were washed twice with PBBS; the final pellet was suspended in 1-2 ml of PBBS. The two cell suspensions were then irradiated at a 15 inch target distance via a General Electric Maximag X-ray unit set at 205 V, 15 mA, with a 1 mm Cu and a 1 mm Al filter. The cells received a total dose of 2,000 rads. Cells were washed twice in PBBS after irradiation and resuspended in Click's Medium (see Appendix A, p. ) at a final concentration of $5 \times 10^6$ viable lymph node cells per ml.

Preparation of Responder Cells

Peripheral lymph nodes were obtained from a normal outbred SD hamster and a single-cell preparation was prepared as described above. Responder cells did not receive irradiation.

Culturing, Pulsing, and Harvesting the Cells

Cell suspensions obtained above were cultured in triplicate in tissue culture plates (Linbro Scientific, Inc., Hamden, Connecticut):
$5 \times 10^5$ responder RML hamster cells alone, $5 \times 10^5$ stimulator CB hamster cells alone, $5 \times 10^5$ RML hamster cells and $5 \times 10^5$ stimulator RML hamster cells, and $5 \times 10^5$ responder RML hamster cells and $5 \times 10^5$ stimulator CB hamster cells.

Culture plates were placed in a humidified incubator set at $37^\circ C$ with a five percent $CO_2$ atmosphere. Twenty-four hours after culturing, 20 µl of heat inactivated FCS were added to each well. Sixteen hours prior to each harvest, the wells were pulsed with 1.0 µCi of $^3$H-thymidine. Cultures were harvested via a multiple automated sample harvester (Otto Niller Company, Madison, Wisconsin) at 48, 72, 96, and 120 hours. Cells were retained on leukocyte Whatman CF/A glass fiber filters placed in minivials (New England Nuclear, Boston, Massachusetts) and dried overnight in a $37^\circ C$ oven. One ml of scintillation fluid (Spectrafilm, Amersham Corporation, Arlington Heights, Illinois) PPO-POPPOP was added to each vial; the amount of radioactivity was counted in a programmable Beckman scintillation counter. The degree of stimulation was expressed as the net counts or ACPM that were co-cultured with stimulator cells.

**Skin Grafting**

Skin grafting was performed via a modified technique described by Billingham and Medawar (14). The donor animal was exsanguinated and the entire dorsal region was shaved and cleansed with a disinfectant soap. A $6 \times 4$ cm portion of the dorsal skin was removed, fastened to a styrofoam board epidermus side down, and the muscularis mucosal layer
was scraped free with a sterilized razor blade. The remaining tissue was stored in cold PBBS for further use.

The host animal was anesthetized with 0.1-0.15 ml of sodium pentobarbitol and the complete dorsal area was shaved and cleansed as described above. A small piece of skin was lifted vertically with a pair of sharp-tipped forceps so as to form a hood. This area was surgically removed to form the graft bed. A section of donor skin was trimmed to approximate the graft bed and sutured into place with 5-0 Dermalon suture. For control purposes the host SD hamsters usually received skin grafts from an inbred SD hamster and an inbred CB hamster. Skin grafts were bandaged with sterile vaseline gauze which was secured by wrapping a small section of cloth sponge around the entire animal and taping it firmly. Care was taken to avoid suffocating the animals.

Skin grafts were first examined on day 5 to confirm vascularization of the donor tissue and then examined daily from day 7 until the graft tissue was accepted or rejected. Graft rejection was considered complete when the donor tissue was necrotic and completely separated from the graft bed. The tissue at this time was dry and did not display hair growth. Acceptance was defined by the presence of healthy donor tissue with hair growth and no signs of necrosis.

Initial grafting experiments utilized donor skin from outbred RML hamsters, inbred CB hamsters, and Lewis rats. The use of graft tissue from an outbred SD hamster served as a technical control because this tissue should be accepted.
**Mitogen Assays**

Lymph nodes were removed from SD hamsters and single-cell suspensions were prepared by the procedure described for the MLR assay. In this case the cells were adjusted to a final concentration of \( 1 \times 10^6 \) viable cells/ml in RPMI-1640 tissue culture medium (see Appendix B, p. 84). Two hundred ul of the cell suspension was added to the wells of a culture plate in triplicate along with the appropriate concentration of mitogen. Dose response and kinetic profiles were obtained by varying the amount of mitogen and harvesting the cells at different times. The mitogens used were ConA and PHA (T-cell mitogens) and LPS (B-cell mitogen).

Twenty-four hours prior to harvesting, the cultures were pulsed with 1.0 \( \mu \)Ci of \(^3\)H-thymidine. The cultures were harvested onto leukocyte filters with a MASH unit. The filters were dried and the amount of incorporated \(^3\)H-thymidine was determined via a Beckman liquid scintillation counter. The degree of mitogenic stimulation was expressed as \( \Delta \text{CPM} = \text{CPM}_{\text{exp}} - \text{CPM}_{\text{control}} \). The net CPM were plotted as a function of mitogen concentration and as a function of time. The optimal concentration and time obtained for each mitogen were used in additional studies. The mitogen studies described for the thymectomized hamsters utilized Click's Medium instead of RPMI-1640. All of the mitogen assays performed in Click's Medium utilized the optimal mitogen dose and time obtained from the earlier studies.
Physical Condition and Survival of Syrian Hamsters Thymectomized and Sham-thymectomized at Birth

Newborn hamsters were thymectomized or sham-thymectomized within 12 hours of birth. After surgery, animals were maintained at room temperature or under a heating lamp until consciousness was restored. All animals regaining consciousness were returned to their litters and observed daily until weaning. Animals were weaned at 21 days of age and segregated by sex. Table 1 contains data representing the survival of the newborn hamsters after surgery. Both the thymectomized and sham-thymectomized groups showed similar survival and mortality data.

The percentage of surgical deaths for thymectomized and sham-thymectomized hamsters was similar. Surgical deaths were probably due to extreme blood loss, heart or lung trauma, and excessive hypothermia. Another cause of neonatal mortality resulted from maternal cannibalism or neglect. Handling of the young would many times cause the mothers to cannibalize their litters. This problem was further compounded by the surgical procedure because it left scents of blood and humans on the neonates. Approximately 70 percent of the total number of animals operated on were lost due to maternal neglect or cannibalism.
Table 1
Survival of Hamsters Thymectomized and Sham-thymectomized at Birth

<table>
<thead>
<tr>
<th>Surgical treatment</th>
<th>No. of litters</th>
<th>Total neonates</th>
<th>No. of surgical deaths*</th>
<th>No. of deaths due to maternal cannibalism or neglect†</th>
<th>No. of animals weaned</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Thymectomy‡</td>
<td>60</td>
<td>602</td>
<td>62 (10.3%)§</td>
<td>418 (69.4%)</td>
<td>58 (9.6%) 63 (10.5%)</td>
</tr>
<tr>
<td>Sham-thymectomy‡</td>
<td>15</td>
<td>151</td>
<td>17 (11.3%)§</td>
<td>100 (66.2%)</td>
<td>15 (9.9%) 22 (14.6%)</td>
</tr>
</tbody>
</table>

*Animals dying during or immediately after surgery; animals not returned to litters.
†Animals found dead after being returned to litters in good health.
‡All animals were thymectomized or sham-thymectomized within 24 hours of birth.
§Percentage of total animals.
Techniques directed toward alleviating this problem were tried with limited success. Distracting the mothers by replacing neonates with bits of food, such as carrots or potatoes, did not work. Removing a litter from a mother until surgery was completed also had little success. One technique appeared to work to some degree, although it was by no means absolute: surgery was performed at night or on weekends when the surgical room was relatively quiet and empty. After the mothers had become acclimated to the new room, one half of the litters were removed for surgery. These animals were swabbed down after surgery with 70 percent EtOH and returned to their litters. The remaining one half of the litters were then removed for surgery. This, when done under the quiet conditions, appeared to have some degree of success.

At weaning, approximately 20 percent of the litters were alive and healthy. The weanlings were segregated, comparably based on sex. There was equal survival at day 21 for thymectomized and sham-thymectomized animals.

Groups of thymectomized and sham-thymectomized animals were observed after weaning for the onset of wasting disease; they were weighed at 30-day intervals. Wasting disease is a condition that develops in many strains of mice and rats after neonatal thymectomy. Wasting is characterized by weight loss, ruffled fur, an unkempt appearance, periorbital edema, and early mortality. Wasting does not develop in thymectomized germ-free mice. This indicates that wasting may be caused by an etiological agent. Thymectomy apparently renders animals more susceptible to infection with this agent or agents.

An easily measured parameter of wasting syndrome was body weight
loss. Wasting disease was associated with less than normal weight gain in thymectomized, wasted mice. Figure 2 shows the body weights of groups of thymectomized, sham-thymectomized, and normal SD hamsters at various intervals after birth. Normal, unoperated animals tended to have slightly higher mean body weights although the difference was not very significant. Mean body weights of thymectomized and sham-thymectomized animals were not significantly different. Slightly higher mean body weights of unoperated animals suggested that surgery had a minimal effect.

Clinical signs of wasting disease were rarely observed. Thymectomized animals generally appeared to be healthy; however, a significant number of thymectomized hamsters died before 90 days of age. This is illustrated in Figure 2. Numbers above the standard deviation bars represent the number of animals weighed on the days indicated. Between day 30 and day 60, approximately 20 percent of the neonatally thymectomized hamsters died; there was no comparable mortality rate in the sham-thymectomized animals. Between day 60 and day 90, another 36 percent of the thymectomized animals died. Although there were no clinical signs of wasting or ill health, thymectomy apparently had some deleterious effect on young adult hamsters. After day 90, the mortality rate in this group leveled off and no additional mortality was seen. The absence of a severe clinical wasting syndrome might indicate that the neonatally thymectomized animals still possessed some level of T-cell immunity; however, the most T-deficient animals may have died before a clinical wasting developed. Early mortality thymectomized hamsters may have eventually showed signs of wasting had
Figure 2. Effect of Thymectomy on the Body Weight and Survival of Young Adult Syrian Hamsters

Each point represents the geometric mean body weight calculated from the experimental groups listed. The bars represent the standard deviation from the geometric mean. The numbers above the bars represent the number of animals used for that point.
they lived longer. Nude mice were usually maintained in dust-free cages with antibiotic or acid water. These procedures extended the life span of these athymic animals.

Characterization of Mitogenic Responses in the Lymph Node Cells from Syrian Hamsters

Peripheral lymph nodes from 60- to 90-day-old normal SD hamsters were removed, pooled, and made into a single-cell suspension as described in Chapter 2. Cells were utilized in mitogen assays in order to determine the optimal dose and time for mitogenic stimulation.

Response to ConA

Some $2 \times 10^5$ viable lymph node cells were added in triplicate to the wells of a culture plate along with concentrations of ConA: 0.5, 1.0, and 2.0 µg. Cells were pulsed, harvested, and an amount of mitogenic stimulation was determined for the three concentrations at 72 hours. Figure 3 shows the degree of stimulation as a function of mitogen concentration. This figure also illustrates that 1.0 µg of ConA in the culture medium provides optimal stimulation at 72 hours. Figure 4 shows the kinetic response of lymph node cells to 1.0 µg of ConA at 48, 72, 96, and 120 hours. There was, however, a significant response at 48 hours. The degree of stimulation steadily increased to an optimum at 96 hours. At 120 hours the response decreased approximately 25 percent compared to the 96-hour response.
Figure 3. Dose Response of Hamsters Lymph Node Cells to Varying Concentrations of ConA

Each point represents the mean CPM calculated from triplicate cultures. The bars represent the range obtained from the above data.

Figure 4. Kinetic Response of Hamster Lymph Node Cells to 1.0 μg of ConA

The points and bars are illustrated as in Figure 3.
Response to PHA-P

Lymph node cells were stimulated with 10, 50, and 100 µg of PHA-P and harvested at 72 hours. The amount of mitogenic stimulation was determined and plotted as a function of mitogen concentration. Figure 5 shows that 50 µg of PHA-P provided optimal stimulation at 72 hours. This concentration was employed in a kinetic study to determine the optimal time of the response. Figure 6 shows that the mitogenic response to 50 µg of PHA-P was maximal at 96 hours. The 72- and 120-hour responses, although significant, were approximately 30 percent lower than the 96-hour response.

Response to LPS

Suspensions of lymph node cells were cultured with 0.1, 1.0, 5.0, and 10.0 µg of LPS. The cells were harvested at 72 hours. The dose response graph is shown in Figure 7. Peak responses were obtained with five and 10 µg of LPS. Because of the toxic nature of LPS, five µg were used in further kinetic studies. The kinetics of the LPS response are shown in Figure 8. The 72- and 96-hour responses were comparable in magnitude with the 96-hour response being slightly greater. Like the ConA and PHA responses, the LPS response also declined on day 5.

Characterization of \textit{In Vivo} and \textit{In Vitro} Cell-mediated Immune Responses in Normal SD Syrian Hamsters

Response to Foreign Skin Grafts

The capability of normal outbred SD hamsters to reject skin grafts was determined by using donor skin from Lewis rats (xenograft)
Figure 5. Dose Response of Hamster Lymph Node Cells to Varying Concentrations of PHA-P

The points and bars are illustrated as in Figure 3.

Figure 6. Kinetic Response of Hamster Lymph Node Cells to 5.0 μg of PHA-P

The points and bars are illustrated as in Figure 3.
Figure 7. Dose Response of Hamster Lymph Node Cells to Varying Concentrations of LPS

The points and bars are illustrated as in Figure 3.

Figure 8. Kinetic Response of Hamster Lymph Node Cells to 5.0 μg of LPS

The points and bars are illustrated as in Figure 3.
and SD hamsters (isograft). The animals (4) received grafts of rat and hamster tissue. The results are presented in Table 2. The xenografts and the isografts were in good condition through days 6 and 7. The grafts were vascularized and visible signs of necrosis were not evident although, by day 10, the xenografts were rejected. They were severely necrotic and completely separated from the graft beds. There was no evidence of hair growth and the tissue had hardened and lost its flexibility. The isografts, on the other hand, showed no signs of necrosis on day 10, had some hair growth, and were confluent with the surrounding tissue.

Isografts served as a technical, surgical control in this experiment. Although the SD hamsters were outbred, they apparently did not differ in their histocompatibility antigens and they were capable of accepting reciprocal grafts. Technical graft rejections can be caused by infection of the graft beds, poor grafting techniques, and physical manipulation of the graft tissue by the animals. In this experiment the rejection of xenogeneic tissue was primarily due to an immunological reaction that was presumably triggered by xenogeneic-reactive T-cells.

Mixed Lymphocyte Reaction

The mixed lymphocyte reaction was an in vitro technique developed to assess the degree of histocompatibility between two individuals. Irradiated lymph node cells from a CB inbred hamster and an outbred SD hamster were used as stimulator cells. Lymph node cells from a normal outbred SD hamster served as responder cells. The results are
<table>
<thead>
<tr>
<th>Animal</th>
<th>Skin graft received</th>
<th>Condition of skin graft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>1♂</td>
<td>Normal outbred hamster</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>Lewis rat</td>
<td>0</td>
</tr>
<tr>
<td>2♂</td>
<td>Normal outbred hamster</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lewis rat</td>
<td>0</td>
</tr>
<tr>
<td>3♂</td>
<td>Normal outbred hamster</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lewis rat</td>
<td>0</td>
</tr>
<tr>
<td>4♂</td>
<td>Normal outbred hamster</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Lewis rat</td>
<td>0</td>
</tr>
</tbody>
</table>

*0 = no visible signs of rejection.
†A = skin graft accepted past 14 days.
‡1 = minimal necrosis of tissue; not a definite rejection.
§2 = severe necrosis of tissue; definite rejection.
shown in Figure 9. At 48 hours there was a minimal response slightly above background levels. At 72 hours, CB-stimulated cells had begun to show a significant proliferative response which continued linearly until it reached an optimum at 96 hours. The proliferative response of the normal SD-stimulated cells remained at background levels for 96 hours although the 120-hour response of this culture increased to a reasonably significant level. This delayed response was possibly due to the presence of minor histocompatibility differences among the SD outbred hamsters. These minor determinants apparently did not play a major role in graft rejections because the outbred SD hamsters readily accepted the reciprocal grafts (Table 2). The proliferative response in the CB-stimulated cultures was quite significant and consistent with published data that a major histocompatibility difference existed between inbred CB hamsters and outbred SD hamsters.

Irradiated stimulator cells cultured alone showed no incorporation of tritiated thymidine, therefore the proliferative response of the cell mixture was due to the incorporation of label by the responder cell populations.

Characterization of the Immune Response in Thymectomized Hamsters: Experiment 1

Anti-HEA Response

Hemagglutination. Age and sex-matched groups of neonatally thymectomized, sham-thymectomized, ATxIR, and normal outbred SD hamsters were immunized with HEA in CFA. Serial plasma bleeds were obtained on days 0, 14, 21, and 28 and assayed for HEA specific
Figure 9. Mixed Lymphocyte Reaction

Kinetics of proliferative response between histoincompatible SD responder and CB stimulator hamster lymph node cells. The points and bars are illustrated as in Figure 3.
Diagram showing the relationship between HOURS and ΔCPM x 10^3. The graph compares Stimulator Cells, CB Cells, and Normal RML Cells over time.
agglutinating antibodies. The hemagglutination data are represented in Figure 10.

Normal hamsters had a good anti-HEA response that reached a peak titer of approximately 12 (log₂) at day 21. The anti-HEA response of sham-thymectomized hamsters was comparable to the response observed in normal animals. The response in sham-thymectomized animals peaked at day 21 with a mean titer of about 10. Thymectomized animals were divided into two groups based on their anti-HEA response. One group, thymectomized responder hamsters, showed a significant anti-HEA response. Optimum titers obtained in the thymectomized responder group were slightly less than the titers seen in normal and sham-thymectomized animals. Thymectomized responder animals represented 20 percent of the thymectomized animals used in this experiment.

The other group, thymectomized nonresponders, had no detectable Ab response. The anti-HEA titers for this group remained constant at about 1.5 (log₂) throughout the experiment. This background HA titer might be due to the presence of natural antibodies or non-specific agglutination of the HEA-SRBC conjugates. ATxIR hamsters also showed no detectable Ab response against HEA.

Ig class specific anti-HEA response. Sera used in determining the hemagglutination titers were analyzed using IEP autoradiography (RIEP) as described in Chapter 2. This technique was more sensitive than hemagglutination and, although it was not a quantitative technique, it identified the Ig class of antibodies produced in response to the antigen. Figures 11 and 12 show representative IEP patterns and the
Figure 10. Kinetics of the Antibody Response of Thymectomized, Sham-thymectomized, and Normal Hamsters to 5.0 μg of HEA-CFA

Each point represents the geometric mean titer calculated from the titers of the individual experimental animals. The bars represent the standard deviation from the mean. The numbers above the bars indicate the number of animals in each experimental group.
Figure 11. Immunoelectrophoresis Pattern (Top) and Corresponding $^{125}$I-HEA Autoradiograph (Below) of Hamster Serum Obtained 21 Days After Inoculation with 5 µg HEA in CFA

The top well contains serum from a sham-thymectomized hamster and the bottom well contains serum from a neonatally thymectomized animal.

Figure 12. Immunoelectrophoresis Pattern (Top) and Corresponding $^{125}$I-HEA Autoradiograph (Below) of Hamster Serum Obtained 21 Days After Inoculation with 5 µg HEA in CAF

The top well contains serum from an ATxIR hamster and the bottom well contains serum from a normal hamster.
corresponding autoradiographic prints using antisera from HEA-sensitized thymectomized and normal hamsters.

Figure 11 shows the RIEP patterns from a neonatally thymectomized (bottom well) and a sham-thymectomized (top well) hamster. Gamma globulins migrated toward the cathode and are visible as the precipitin lines at the left end of the IEP pattern. Directly below the IEP pattern is the corresponding autoradiographic print. Sera in the bottom well bound the radioactive antigen and two precipitin lines are evident. The precipitin line closest to the cathode corresponds to the IgG\textsubscript{2} class; the adjacent, faster precipitin line represents the IgG\textsubscript{1} class. Thus the sham-thymectomized hamster elicited both an IgG\textsubscript{1} and an IgG\textsubscript{2}-specific anti-HEA response. Sera from the thymectomized animal showed no antigen-binding and, thus, no detectable antibody response against HEA.

Figure 12 is another RIEP pattern. Serum in the bottom well is from a normal SD hamster whereas serum in the top well is from an ATxIR animal. The RIEP pattern revealed IgG\textsubscript{1} and IgG\textsubscript{2} anti-HEA in immunized normal sera whereas ATxIR animals showed no detectable Ag binding.

Sera from all the experimental animals were assayed for the Ig class containing anti-HEA activity. Table 3 is a summary of the day 28 anti-HEA response which shows that normal and sham-thymectomized hamsters produced IgG\textsubscript{1} and IgG\textsubscript{2} antibodies in response to HEA. Similar results were found with thymectomized responder animals; however, thymectomized nonresponder and ATxIR animals had no detectable Ag binding. The RIEP data correlated directly with the hemagglutination data. All
Table 3

Ig Class Specificity of Anti-HEA Response in Thymectomized and Normal Syrian Hamsters: Day 28

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>IgG₁</th>
<th>IgG₂</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td>8/8†</td>
<td>8/8</td>
<td>0/8</td>
</tr>
<tr>
<td>Sham-thymectomized</td>
<td>11/11</td>
<td>11/11</td>
<td>0/11</td>
</tr>
<tr>
<td>Thymectomized responders</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Thymectomized nonresponders</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>ATxIR</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

*All animals were operated on within 12 hours of birth.
†Number of animals responding/number of animals tested.

Sera with the HEA-agglutinating antibodies had IgG₁ and IgG₂ anti-HEA as detected by RIEP. Conversely, sera without agglutinating antibodies did not bind ¹²⁵I-HEA.

Allograft Response

Experimental animals used in the previous study were grafted with skin from a CB inbred hamster in order to assess the cell-mediated immune response. Grafts were observed for a three-week period. The condition of the grafted tissue was closely recorded. Table 4 summarizes the results obtained from the allograft response.

Sham-thymectomized hamsters rejected the CB graft with a mean survival time (MST) of 10.3 ± 1.5 days. Neonatally thymectomized hamsters rejected their CB grafts in 13.5 ± 2.4 days. The thymectomized
Table 4
Allograft Response in Thymectomized, Sham-thymectomized, and ATxIR Syrian Hamsters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of grafts accepted/ no. of animals tested</th>
<th>Mean survival time ± S.D. (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-thymectomized</td>
<td>0/6</td>
<td>10.3 ± 1.5 (9-12)</td>
</tr>
<tr>
<td>Thymectomized</td>
<td>0/11</td>
<td>13.5 ± 2.4 (11-16)</td>
</tr>
<tr>
<td>ATxIR</td>
<td>3/3</td>
<td>&gt; 21</td>
</tr>
</tbody>
</table>

The group included HEA thymectomized responders (8) and thymectomized non-responders (3) animals. There was no significant difference between the MST values obtained for these two groups.

The difference between the MST of sham-thymectomized and non-thymectomized hamsters was not significant. Although the MST for thymectomized hamsters was slightly higher than the MST for sham-thymectomized animals, the standard deviations and ranges overlapped. ATxIR hamsters, however, did not reject their skin grafts; the grafts appeared healthy and in good condition three weeks after the grafts had been introduced.

These experiments showed that neonatally thymectomized hamsters were capable of rejecting allografts. This response was absent in ATxIR animals.

Mitogen Response

Mitogen assays for the aforementioned experimental animals were set up but, due to contamination of the culture medium, no data were obtained.
Characterization of the Immune Response in Thymectomized Hamsters: Experiment 2

The experiments described in Experiment 1 were repeated on four normal, four sham-thymectomized, and six neonatally thymectomized hamsters.

Anti-HEA Response

The animals were immunized with HEA in CFA and the immune sera were assayed for the presence of anti-HEA antibodies. The Anti-HEA response of these animals 21 days after immunization is summarized in Table 5. Normals and sham-thymectomized animals demonstrated high titers of anti-HEA antibody. Thymectomized animals were segregated into two groups: four responders and two nonresponders as observed in Experiment 1. Thymectomized responder animals demonstrated a normal anti-HEA response while the response of the thymectomized nonresponder animals did not increase above background levels. Data obtained from RIEP correlated with the hemagglutination data. This is also summarized in Table 5.

Allograft Response

The animals described above were tested for an allograft response with CB hamster skin, the results of which are summarized in Table 5. Sham-thymectomized and thymectomized responder animals rejected their skin grafts in comparable time although the thymectomized nonresponder animals accepted their skin grafts for greater than 21 days. Therefore, in this experiment thymectomized nonresponder animals accepted allografts. This result differed from the first experiment in which thymectomized nonresponder animals rejected allografts.
Table 5

Anti-HEA and Allograft Responses in Thymectomized and Normal Syrian Hamsters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals tested</th>
<th>Anti-HEA response: day 21</th>
<th>Allograft rejection</th>
<th>MST ± S.D. (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean HA titer ± S.D. (log₂)</td>
<td>IgG₁</td>
<td>IgG₂</td>
</tr>
<tr>
<td>Normals</td>
<td>4</td>
<td>10.0 ± 4.0</td>
<td>4/4*</td>
<td>4/4</td>
</tr>
<tr>
<td>Sham-thymectomized</td>
<td>4</td>
<td>8.0 ± 1.4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Thymectomized responders</td>
<td>4</td>
<td>9.3 ± 4.6</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Thymectomized nonresponders</td>
<td>2</td>
<td>1.5 ± 0.7</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

*Number of animals responding/number of animals tested.
†Not determined.
‡Greater than 21 days.
Mitogen Response

Table 6 shows the ConA, PHA, and LPS responses of lymph node cells from the animals described in the previous experiment. Normal, sham-thymectomized, and thymectomized responder animals all demonstrated a normal T-cell response to ConA. Although thymectomized nonresponder animals accepted allografts and failed to make anti-HEA Abs, one animal demonstrated a normal ConA response while the second animal demonstrated a slightly diminished response. Similar results

Table 6

Mitogenic Responses in Normal, Thymectomized, and Sham-thymectomized Syrian Hamsters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response to mitogens</th>
<th>72 hours CPM x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bkgd*  ConA†</td>
<td>PHA‡</td>
</tr>
<tr>
<td>Normal</td>
<td>6                510</td>
<td>328</td>
</tr>
<tr>
<td>Sham-thymectomized</td>
<td>3                578</td>
<td>538</td>
</tr>
<tr>
<td></td>
<td>2                655</td>
<td>380</td>
</tr>
<tr>
<td>Thymectomized responders</td>
<td>6                640</td>
<td>532</td>
</tr>
<tr>
<td></td>
<td>18               605</td>
<td>476</td>
</tr>
<tr>
<td></td>
<td>2                666</td>
<td>550</td>
</tr>
<tr>
<td>Thymectomized nonresponders</td>
<td>1                158</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>2                530</td>
<td>274</td>
</tr>
</tbody>
</table>

*CPM without mitogens.
†Concanavalin A = 1 µg/ml.
‡Phytohemagglutinin = 50 µg/ml.
§Lipopolysaccharide = 5 µg/ml.
were obtained with PHA.

Recent findings by Blasecki indicated that the ConA and PHA reactive cells in hamsters were thymus derived (18), therefore the elicitation of a ConA and PHA proliferative response in the thymectomized nonresponder animals suggested that T-cells or mitogen reactive T-cell precursors are present in these animals. These mitogen reactive cells are not, however, capable of functioning as helper cells in an anti-HEA response.
Chapter 4

DISCUSSION

Removal of the thymus shortly after birth resulted in impaired immunological responses in mice, rats, and rabbits (1, 47, 57-59, 65, 68, 70). The reported immunological deficiencies affected the humoral Ab and the cell-mediated immune responses. The effects associated with neonatal thymectomy have been attributed to a depletion of mature, functional T-cells. Studies dealing with the role of the thymus in Syrian hamsters have yielded inconsistent and conflicting data (87, 90, 91). The results obtained in the present study clearly demonstrated the requirement for thymus-derived lymphocytes in an anti-HEA Ab response; however, neonatal thymectomy did not appear to produce as severe an effect on the allograft response. ATxIR animals demonstrated an absence of an anti-HEA response and an allograft response. These findings indicated that neonatal thymectomy results in only a partial, and possibly a selective, depletion of functional T-cells.

Absence of Wasting Disease in Neonatally Thymectomized Hamsters

In the present study, neonatally thymectomized hamsters did not develop post-thymectomy wasting disease. Although approximately 50 percent of the neonatally thymectomized and 20 percent of the sham-thymectomized hamsters died within three months after weaning, signs of wasting were not evident prior to or at the time of death. Thymectomy
apparently resulted in the early mortality of these animals. It is conceivable that these animals may have died before clinical signs of wasting developed. They may have been the most T-depleted of the thymectomized hamsters and, thus, died early. These findings were in sharp contrast to Sherman's description of a severe wasting disease following thymectomy in hamsters (89). The discrepancy between these findings was not easily resolved (other authors had not observed wasting in thymectomized hamsters [43,87]). Sherman's finding remains an isolated observation that has not been confirmed.

Relatively little is known about wasting disease. Because neonatally thymectomized germ-free mice did not develop this condition, it was believed to be caused by an infectious agent (74, 77). Wasting may be an indirect result of thymectomy due to a relative state of immunological incompetence. Thymectomy can curtail the development of an immune response in the host, thus permitting the establishment of infectious agents.

The absence of recognizable wasting disease in thymectomized hamsters indicated that some degree of T-cell immunity was still present in these animals. When nonwasted thymectomized rats were tested for a DTH response, the animals were separable into two groups: responders and nonresponders (27). The presence of responder and nonresponder groups of thymectomized animals was similar to the results obtained from the thymectomized hamsters when tested at the Ab level.

**Anti-HEA Response in Thymectomized Hamsters**

Although thymectomized hamsters did not develop wasting disease,
they demonstrated a severe deficiency in the production of Abs against HEA. HEA-CFA was an excellent antigen in normal hamsters. It elicited the simultaneous production of IgG$_1$ and IgG$_2$ Abs (27). Neonatal thymectomy resulted in the abrogation of a detectable anti-HEA response.

Studies in other animals have revealed a T-cell requirement for Ab production against complex protein antigens (2, 19, 80). The antibody responses to BSA, HSA, and HEA (serum albumins) were shown to decrease after neonatal thymectomy (2, 3, 19, 80). The present study showed that T-cells are required for an anti-HEA response in hamsters.

Both the IgG$_1$ and IgG$_2$ anti-HEA Abs were absent after immunization of thymectomized hamsters. In mice and rats, the IgG$_1$ class demonstrated a greater T-cell dependency than other Ig classes. Torrigiani (104) and Arnason (3) reported that the antibody responses to HSA and HEA, respectively, were predominantly IgG$_1$. The T-cell requirement for these IgG$_1$ responses was demonstrated after neonatal thymectomy resulted in a severe reduction in the IgG$_1$ Abs. The relative T-dependence of IgG$_1$ and IgG$_2$ in hamsters was not evaluated in this study due to the complete absence of anti-HEA Ab in each Ig class. Perhaps a different sensitization procedure (varying amounts of HEA, different route of injection, different Ag) could have assessed any differences in the T-dependency of the hamster IgG$_1$ and IgG$_2$ Ab classes.

Studies by Coe et al. (27, 88) have revealed an interesting phenomena with respect to the anti-HEA response in hamsters. Hamsters immunized with HEA-CFA elicited an IgG$_1$ and IgG$_2$ specific response whereas those inoculated with the antigen without CFA produced only IgG$_1$
Abs. Repeated immunizations failed to elicit an IgG₂ response. Furthermore, when these same animals were challenged with HEA-CFA, they demonstrated an anamnestic IgG₁ response while an IgG₂ response was not detectable. The IgG₂ class of Igs had been made tolerant to HEA. This split tolerance phenomena implied that a complex network of cells might be regulating the Ab response against HEA. A reasonable hypothesis would predict that this response was under T-cell regulation; however, the additional regulatory role of macrophages or B-cells could not be ruled out. The observation of split tolerance and the results of the present study suggested that the anti-HEA response in hamsters was under T-cell regulation.

Not all of the thymectomized hamsters demonstrated absent anti-HEA responses after immunization. Approximately 20 percent of the thymectomized hamsters demonstrated significant anti-HEA responses. Apparently these thymectomized responder animals possessed helper T-cells that could function in an Ab response. Such T-cells might arise from incomplete thymectomy or from seeding of functional T-cells prior to thymectomy.

Although thymectomized responder animals elicited anti-HEA Abs after immunization, the kinetics of this response was much slower compared to normal animals. This indicated that a partial depletion of T-cells may have been achieved. The response plateaued on day 21; it did not reach the same optimum titers seen in normal and sham-thymectomized animals. One explanation could be that a limited number of helper T-cells were present in these animals and that this resulted in the slower Ab kinetics observed.
The absence of an anti-HEA response in thymectomized hamsters suggested that the specific helper T-cells involved in this response were depleted. Although direct evidence for T-cell subpopulations was lacking in hamsters, the absence of a T-dependent Ab response suggested that a hamster's equivalent Ly 1⁺ helper T-cell (see Figure 1, p. 19) was removed by thymectomy. The progenitors of Ly 1⁺ helper cells in mice were the Ly 123⁺ amplifier cells. These were especially susceptible to the effects of thymectomy (54). The T-cell deficiency observed in thymectomized hamsters might be attributed to the removal of the hamster equivalent of the Ly 123⁺ precursor cells.

If a small number of the Ly 123⁺ cells were to seed the peripheral lymphoid tissues prior to thymectomy, an animal would still possess a source of functional T-cells. The thymus of hamsters was lymphoidal by the thirteenth day of gestation and the lymph nodes and spleen were lymphoidal at birth (93), therefore some degree of thymic seeding may have occurred prior to birth. There was the possibility that neonatal thymectomy, no matter how early it was performed, would not result in an absolute depletion of functional T-cells.

**Allograft Response in Thymectomized Hamsters**

Despite the apparent absence of helper T-cells in neonatally thymectomized hamsters, demonstrable DTH reactions were observed against an allogeneic tissue graft. The capacity to reject foreign tissue has long been considered a T-dependent phenomena (46). (Experimental manipulations with alloreactive Abs have resulted in T-independent graft rejections known as white grafts.)
Hamsters were somewhat unique in that the immune response to alloantigens could be measured by graft rejection, MLR, and GVHR in the consistent absence of circulating Abs. This would eliminate the possibility that an Ab-dependent graft rejection was responsible for the thymectomized animals' capacity to reject skin grafts. Graft rejection observed in these hamsters must therefore be attributed to alloreactive T-cells.

In contrast to the neonatally thymectomized hamsters, AtxIR hamsters failed to reject skin grafts. The absence of an allograft response indicated that a greater degree of T-cell depletion was achieved in these animals. It is conceivable that ATxIR hamsters would be more representative of the nude mouse model. These hamsters were thymectomized as young adults and subjected to lethal irradiation. The dosage destroyed the bone marrow and a great majority of the blood-born cells (including T- and B-cells). The irradiated animals were repopulated with syngeneic bone marrow cells that reconstituted the destroyed marrow and served as a source of pluripotential stem cells. These stem cells could migrate to different hematopoietic microenvironments (the thymus, bursa-mammalian equivalent) and mature into B-cells, T-cells, macrophages, and erythrocytes, eventually repopulating the entire lymphatic and circulatory systems. In the absence of the thymus, however, differentiation of stem cells into post-thymic precursor cells would be prevented. These animals should theoretically be completely void of T-cells and, therefore, they should not demonstrate classical T-dependent immune reactions. This was corroborated by the present findings.
Possible Models of Functional T-cell Development in Hamsters

The varying degree of immunological defects observed after neonatal thymectomy in hamsters suggested two possible explanations for these results: (1) a quantitative model for T-cell development and (2) a qualitative or temporal model for T-cell development. The quantitative model of T-cell development suggested that functionally heterogeneous T-cell populations were continuously seeded from the thymus. A critical number of cells would be required in order to detect a particular immune response: Ab production, skin graft rejection, mitogen responsiveness. Some assays (Ab production) might have required more T-cells than an assay such as graft rejection. Perhaps the difference in these assays represents variations in the immunogenicity of the antigen--that a large amount of a potent immunogen (skin graft) would require fewer T-cells than a small amount of a weaker immunogen (HEA).

Mice studies have shown that approximately 60 percent of all T-cells possessed alloreactivity whereas a minor proportion of the T-cells were responsive to soluble antigens (36). If these data were valid in hamsters, any treatment resulting in an overall depletion of T-cells would have had a more profound effect on the HEA response than on the allograft response.

A small percentage (10 percent) of neonatally thymectomized hamsters demonstrated impaired humoral Ab and allograft responses. In line with the quantitative model, these animals would have been more depleted of T-cells. One would expect these animals to demonstrate
lower levels of T-cell mitogen responsiveness, but near normal ConA and PHA reactivity was observed. One explanation might be that seeded, undifferentiated T-cell precursors not detectable by Ab or skin graft assays were capable of responding to ConA or PHA. On the other hand, the mitogen response might represent the most sensitive T-cell assay (i.e., 100 percent of the T-cells could respond to mitogens, 50 percent of the T-cells could respond to allografts, 10 percent of the T-cells were detected with a specific Ab response [hypothetical percentages]). Studies using limiting dilution analysis and reconstitution of AtxIR hamsters would help to solve this dilemma.

An alternative hypothesis would be that the anti-HEA and allograft responses represented qualitatively different immune responses. This model suggested that immunological functions developed at a precise time, and would be dependent on the functional nature of the T-cells present.

Data obtained in this study allowed for the construction of a theoretical sequence of T-cell functions during the development of the immune system. Three groups of animals emerged based on observed immunological capabilities (Table 7): (1) sham-thymectomized and thymectomized responder animals demonstrated mitogen responsiveness, capacity to reject an allograft, and an anti-HEA Ab response, (2) thymectomized nonresponder hamsters did not produce Ab against HEA, but they were capable of rejecting allografts and responding to ConA and PHA, and (3) thymectomized nonresponder, nonrejcctor hamsters failed to produce Ab to HEA; they were incapable of rejecting an allograft. These animals demonstrated near normal ConA and PHA responsiveness.
Table 7
Classification of Thymectomized Syrian Hamsters

<table>
<thead>
<tr>
<th>Effect observed</th>
<th>Treatment</th>
<th>Anti-HEA response</th>
<th>Allograft rejection</th>
<th>Mitogen response</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Sham-thymectomized +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Nonthymectomized</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Moderate</td>
<td>Nonthymectomized</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Severe</td>
<td>Nonthymectomized</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>AtxIR</td>
<td>0</td>
<td>0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

ATxIR hamsters were also classified as nonresponders, nonjector animals. The mitogen responsiveness of these animals was not determined. The ATxIR hamsters conceivably represented the most T-depleted models in this study. One could predict reduced levels of ConA and PHA reactivity. If bone marrow cells could respond to ConA or PHA, these animals would demonstrate mitogen reactivity. Hart et al. (51) reported that, although hamster thymocytes responded to ConA and PHA, bone marrow cells were refractory to these T-cell specific mitogens (18). This finding implied that residence in the thymus was necessary for the development of ConA and PHA reactivity. The presence of a significant mitogen response in thymectomized hamsters indicated that some degree of thymic seeding had probably occurred.

All of the experimental groups listed in Table 7 demonstrated mitogen responsiveness when tested with ConA or PHA. This suggested that mitogen reactivity might be one of the first properties to appear during the development of T-cells. These mitogen-reactive cells were
the earliest to seed the peripheral tissues and, thus, they were most resistant to the effects of neonatal thymectomy.

The none and moderate groups demonstrated skin graft responses. This suggested that alloreactive cells developed later than mitogen-reactive cells. Only 10 percent of all the neonatally thymectomized animals tested demonstrated an abrogated allograft response. The relative degree of difficulty in producing a nonrej ector animal implied that alloreactive T-cells were seeded at an early time. Solomon demonstrated that thymocytes obtained from newborn hamsters were capable of responding in an MLR (93).

The relative ease observed in abrogating an anti-HEA response implied that helper T-cells developed at a later time than the mitogen-responsive and alloreactive T-cells. Two thirds of the neonatally thymectomized animals demonstrated no detectable anti-HEA Abs. Because these animals all possessed mitogen-reactive cells, it appeared that they were incapable of differentiating into T-helper cells. This maturational step might have required the direct presence of the thymus or a thymic hormone. Stutman has shown that the differentiation of PTP cells into functional T-cells required the presence of a thymic hormone (97). If this hormone appeared later than birth, all of the immune functions requiring this hormone would be absent until the thymic factor was released. It is obvious that removal of the thymus would remove the source of PTP cells and the thymic hormone, thereby preventing the further maturation of these cells. Some PTP cells may have remained "frozen" at an immature stage. This would explain why thymectomized hamsters revealed varying degrees of immunological responsiveness.
The thymus in hamsters plays a vital role in early life in regard to the production and seeding of PTP cells. These cells populated the peripheral tissues and, under a hormonal influence from the thymus, matured into immunologically active T-cells.

Conclusions

The present study has demonstrated that hamsters were capable of eliciting many of the immunological responses (helper function in Ab production, allograft and xenograft rejection, MLR, and mitogen responsiveness) that have been attributed to functional T-cells in mice. Neonatal thymectomy and adult thymectomy followed by irradiation and bone marrow repopulation were shown to result in impaired anti-HEA and skin allograft responses. Neonatal thymectomy resulted in varying degrees of immunological impairment with the anti-HEA response being primarily affected. A majority Ab-nonresponder animals demonstrated normal allograft, ConA, and PHA responses.

These findings indicate that neonatal thymectomy in Syrian hamsters does not result in an absolute depletion of T-cells. Seeding of T-cells must therefore occur before or shortly after birth in hamsters. Consistently early thymectomy might help to alleviate the heterogeneous effects of thymectomy seen in the present study.

In the present study a significant proportion of thymectomized hamsters died after weaning and prior to 90 days of age. Sham-thymectomized hamsters did not show this early mortality. Therefore, it might be necessary to maintain all thymectomized animals in somewhat more germ-free conditions. Acid or antibiotic water and the use of
dust-free cages placed in a quarantined room might prolong the lives of thymectomized animals.

It would have been of interest to study more ATxIR animals since these animals appear to be more T-cell depleted. These animals could also serve as models for reconstitution experiments involving fractionated T-cells. Fractionation could be achieved on nylon wool columns or density gradients. Analysis of T-cells in this manner could contribute to the knowledge of the T-cell system in Syrian hamsters.

**Summary**

The present study has revealed the following about the immune response of Syrian hamsters:

1. Hamsters were capable of eliciting both an IgG\textsubscript{1} and IgG\textsubscript{2} specific Ab response against HEA, a T-dependent Ag.

2. Hamsters were capable of rejecting both allografts and xenografts in a period of from 9-12 days.

3. Hamster lymph node cells elicited a proliferative response when stimulated by the murine T-cell specific mitogens, ConA, and PHA.

4. Hamster lymph node cells were capable of eliciting an MLR.

5. Neonatal thymectomy on hamsters had the following effects:
   a. Severe impairment of the IgG\textsubscript{1} and IgG\textsubscript{2} anti-HEA response.
   b. Minimal impairment of the allograft response.
   c. No impairment of the ConA or PHA responses.

6. ATxIR on hamsters produced the following effects:
   a. Severe impairment of the IgG\textsubscript{1} and IgG\textsubscript{2} anti-HEA response.
b. Severe impairment of the allograft response.

c. Effect on mitogen responses not determined.


27. Coe, J. E. The Immune Response in the Hamster. III. Selected Induction of Concomittant Formation and Unresponsiveness in the 7S\textsubscript{Y1} and 7S\textsubscript{Y2} Globulins with Soluble Hen Egg Albumin. Imm. 21:175(1970).


Alserver's Solution--1 liter

NaCl 4.20 gm.
Sodium Citrate 9.12 gm.
-Bring to 900 ml with distilled water
-Adjust pH to 6.1 with 10 percent citric acid
-Autoclave for 15 minutes
-When cool, add 30 ml of sterile 20.5 percent glucose solution

Buffer A--used in HEA sensitization of SRBCs

21.5 ml M/15 Na₂HPO₄
4.9 ml M/15 KH₂PO₄

Click's Medium

<table>
<thead>
<tr>
<th>Stock ingredient</th>
<th>Stock/100 ml Click's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled H₂O</td>
<td>71.5 ml</td>
</tr>
<tr>
<td>Hank's balanced salt solution (10x)</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>MEM essential amino acids (50x)</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>MEM nonessential amino acids (100x)</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Nucleic acid precursors (100x)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>MEM vitamins (100x)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Sodium pyruvate (100 mM)</td>
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</tr>
<tr>
<td>L-glutamine (200 mM)</td>
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</tr>
<tr>
<td>NaOH (2N)</td>
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<tr>
<td>Penicillin-streptomycin (100x)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>2-Mercaptoethanol (0.1 M)</td>
<td>0.04 ml</td>
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<tr>
<td>Sodium bicarbonate (7.5 percent)</td>
<td>1.8 ml</td>
</tr>
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</table>

Phosphate Buffered Balanced Salt (PBBS)--5 liters

<table>
<thead>
<tr>
<th></th>
<th>1.00 gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>5.75 gm.</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.70 gm.</td>
</tr>
<tr>
<td>KCl</td>
<td>1.60 gm.</td>
</tr>
<tr>
<td>NaCl</td>
<td>36.00 gm.</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>1.00 gm.</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>Phenol Red (1 percent stock)</td>
<td>5 ml.</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.00 gm.</td>
</tr>
</tbody>
</table>

-pH to 7.3 and sterilze by pressure filtration
Phosphate Buffered Saline (PBS)---1 liter

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 6.818 \text{ gm.} \\
\text{KH}_2\text{PO}_4 & \quad 2.540 \text{ gm.} \\
\text{MgCl} & \quad 4.240 \text{ gm.} \\
\end{align*}
\]

-pH to 7.2 and autoclave
APPENDIX B
Radiolabelled $^{125}$I-HEA and Rabbit anti-Whole Hamster Sera were gratefully supplied by Dr. J. E. Coe of the Rocky Mountain Laboratory.

Concanavalin A, lipopolysaccharide, Click's medium, and RPMI-1640 medium were gratefully supplied by Dr. K. B. Von Eschen of the Rocky Mountain Laboratory.