Cellular interactions in the in vitro immune response
by David Paul Aden

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
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Abstract:
Spleen cell cultures from athymic (nude) mice were found to be unresponsive to sheep erythrocytes (SE) in vitro. The addition of 5 X 10 thymus cells from Balb/c or littermate animals to nude spleen cell cultures enabled these cultures to respond to SB. An in vitro response to SB could also be obtained by addition of normal spleen cells from Balb/c or littermate mice. As few as 2.4 X 105 normal spleen cells established an immune response in nude spleen cell cultures. Cell cultures prepared from nude mice grafted with thymus glands 2-5 months prior to being used as spleen cell donors responded to SB as well as Balb/c spleen cell cultures. Using various combinations of adherent and nonadherent cells from nude and Balb/c spleens, the adherent cell population of nudes was seen to be functional in the immune response to SB. Heterologous thymus and spleen cells from rats failed to establish an immune response to SB in nude spleen cell cultures. Thymus cells were observed to be radiation sensitive, with a dose of 400 rads effectively inhibiting their ability to establish an immune response to SB in nude spleen cell cultures. Thymus-derived spleen cells, obtained from mice lethally irradiated and injected with thymus cells, were also found to be radiation sensitive, but not as sensitive as thymus cells obtained directly from the thymus. Nude spleen was observed to have functional bone marrow-derived cells because nude spleen cell cultures responded in vitro to lipopolysaccharide (LPS) when whole heat killed E. coli cells (C-LPS) were used as the antigen. LPS has previously been reported to be an antigen that does not require the participation of thymus-derived cells to give an immune response. The obtaining of an immune response to C-LPS in nude spleen cell cultures and the ability to establish an immune response to SB with thymus cells indicated that while a deficiency of thymus-derived cells exists in nude mice, they have functional bone marrow-derived cells. Suppression of the immune response to SB was obtained by an in vitro treatment of spleen cells for four hours or by adding to cell cultures highly specific, high titered heterologous anti-IgM heavy chain antiserum. In vitro treatment (four hours) of Balb/c spleen cells totally inhibited the immune response to SB, and treatment of nude spleen cells inhibited the ability of thymus cells to establish an immune response to SB. Treatment of thymus cells with antiserum did not affect their ability to establish an immune response to SB in nude spleen cell cultures. Furthermore, in vivo suppression of Balb/c mice from birth totally suppressed the response to SB in cell cultures prepared from their spleens, but did not affect the ability of their thymus cells to establish an immune reponse to SB in nude spleen cell cultures.

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ABSTRACT

Spleen cell cultures from athymic (nude) mice were found to be unresponsive to sheep erythrocytes (SE) in vitro. The addition of 5 X 10^7 thymus cells from Balb/c or littermate animals to nude spleen cell cultures enabled these cultures to respond to SE. An in vitro response to SE could also be obtained by addition of normal spleen cells from Balb/c or littermate mice. As few as 2.4 X 10^5 normal spleen cells established an immune response in nude spleen cell cultures. Cell cultures prepared from nude mice grafted with thymus glands 2-5 months prior to being used as spleen cell donors responded to SE as well as Balb/c spleen cell cultures. Using various combinations of adherent and nonadherent cells from nude and Balb/c spleens, the adherent cell population of nudes was seen to be functional in the immune response to SE. Heterologous thymus and spleen cells from rats failed to establish an immune response to SE in nude spleen cell cultures. Thymus cells were observed to be radiation sensitive, with a dose of 400 rads effectively inhibiting their ability to establish an immune response to SE in nude spleen cell cultures. Thymus-derived spleen cells, obtained from mice lethally irradiated and injected with thymus cells, were also found to be radiation sensitive, but not as sensitive as thymus cells obtained directly from the thymus. Nude spleen was observed to have functional bone marrow-derived cells because nude spleen cell cultures responded in vitro to lipopolysaccharide (LPS) when whole heat killed *E. coli* cells (C-LPS) were used as the antigen. LPS has previously been reported to be an antigen that does not require the participation of thymus-derived cells to give an immune response. The obtaining of an immune response to C-LPS in nude spleen cell cultures and the ability to establish an immune response to SE with thymus cells indicated that while a deficiency of thymus-derived cells exists in nude mice, they have functional bone marrow-derived cells. Suppression of the immune response to SE was obtained by an in vitro treatment of spleen cells for four hours or by adding to cell cultures highly specific, high titered heterologous anti-IgM heavy chain antiserum. In vitro treatment (four hours) of Balb/c spleen cells totally inhibited the immune response to SE, and treatment of nude spleen cells inhibited the ability of thymus cells to establish an immune response to SE. Treatment of thymus cells with antiserum did not affect their ability to establish an immune response to SE in nude spleen cell cultures. Furthermore, in vivo suppression of Balb/c mice from birth totally suppressed the response to SE in cell cultures prepared from their spleens, but did not affect the ability of their thymus cells to establish an immune reponse to SE in nude spleen cell cultures.

INTRODUCTION

In vitro cell culture techniques have become useful systems in studies of cell interactions in the immune response. By controlling the numbers and types of cells that are allowed to interact, at least three cell types have been shown to be required to generate an in vitro response to heterologous erythrocytes: an adherent cell, a thymus-derived nonadherent cell (T cell), and a bone marrow-derived nonadherent cell (B cell) (1). The bone marrow-derived lymphocyte produces the humoral antibody and is triggered by a cooperating thymus-derived lymphocyte to do so.

Cultures prepared from the spleens of thymus-derived mice have been useful in distinguishing the roles of T cells and B cells. Thymus-deprived animals have been prepared either by neonatal thymectomy (2) or by adult thymectomy followed by total body irradiation and reconstitution with bone marrow cells (3,4). These thymus-deprived mice are probably not entirely depleted of T cells due to: (a) cell seeding from the thymus prior to its removal at birth, (b) the relative radioresistance of some T cells (5), and (c) the presence of T cells in the bone marrow preparations used to reconstitute irradiated mice (5,6).

The above objections are largely alleviated by using cell cultures prepared from the congenitally athymic (nude) mouse in studies of cell interaction. Nude mice were first described by Flanagan (7)

and were later shown by Pantelouris (8) to lack normal thymuses. In nude mice the blood lymphocyte count is low (9) and the thymus-dependent areas of lymph nodes, spleen, and Peyer's patches are deficient in lymphocytes (10) and cells bearing the theta antigen (11). The experiments of Wortis, Nehlsen, and Owen (12) suggest that nudes do not lack the precursors for thymocytes but suffer from a defect of the thymic epithelium. The congenitally athymic mouse would thus seem to be a useful tool in in vitro studies of cell interaction.

The in vivo response to some antigens, such as lipopolysaccharide (LPS) (13,14), pneumococcal polysaccharide (SIII) (14,15), polyvinylpyrrolidone (13), and polymerized flagellin (16) does not require thymus-derived cells. Most of these studies have used thymus-deprived mice in the cases of LPS and SIII. Manning, Reed, and Jutila (14) have shown that nude mice respond to these antigens while failing to respond to heterologous erythrocytes (17).

Heterologous erythrocytes have been shown to elicit a good immune response in in vitro cell cultures (18,19,20). However, only a limited number of other antigens have been used successfully in vitro. These include protein antigens, such as rabbit Fab'$_2$ (21) and POL (22), SIII (23), and haptens conjugated to erythrocytes (24).

The cooperation of specific B cells with specific T cells in the initiation of an immune response is believed to involve specific

immunoglobulins, and these immunoglobulins are probably on the surface of the lympoid cells (25). It is generally agreed that many of the peripheral B cells, such as those in the spleen, bear surface immunoglobulins. There is conflicting evidence, however, as to whether T cells are essentially lacking in surface immunoglobulins (26,27) or bear them in low but detectable numbers (28,29).

The main approach for determining the biological significance of these surface immunoglobulins has been based upon utilization of a wide selection of antisera directed against whole immunoglobulins or their constituent chains. It has been reported, for example, that the plaque-forming response of cells cultured in vitro can be suppressed by treatment with antisera against whole gamma globulin, and specific heavy and light chains (28,29,30). In addition, mice treated from birth with heterologous antiserum against μ chain are absolutely suppressed in their ability to produce IgM, as reflected in serum immunoglobulin levels and direct plaque formation (30).

In addition to being able to stimulate spleen cell cultures from thymus-deprived mice with other lympoid cells, it has been reported that supernatants from thymus or spleen cells were capable of reconstituting these cultures. Summaries of the literature involving soluble mediators of immune reactions and cell interaction in the immune response are found in the reviews by Talmage, Radovich, and Hemmingsen (1), Claman and Mosier (31), Bloom (32), and Miller (33).

INTRODUCTION TO THESIS PROBLEM

A culture system for cell suspensions from mouse spleen was described by Mishell and Dutton in 1966 (18) which provides adequate conditions for in vitro immunization on initial exposure to heterologous erythrocytes. The in vitro response closely parallels that observed in vivo with respect to size, early kinetics, and antigen dose. The two main respects in which cultured cells differ from observations with intact animals are in the increased ability of cells in culture to discriminate between different varieties of homologous erythrocytes and that the in vitro response does not appear to be limited by the mechanisms that regulate the in vivo response.

It was thus decided that the in vitro system utilizing cells from the nude mouse would be a good approach toward studying cell interaction. Previous to this time, Reed and Jutila (17) had demonstrated that nudes failed to respond to heterologous erythrocytes in vivo. The following questions were initially posed for experimentation: (a) Will spleen cells from nude mice respond to heterologous erythrocytes under cell culture conditions? (b) If nude mouse spleen cells do not respond to heterologous erythrocytes, will the addition of deficient cells (thymus cells obtained directly from the thymus) interact and cooperate with nude spleen cells to give a response to sheep erythrocytes (SE)? (c) Will the spleen cells from normal animals cooperate with nude spleen cells to give an immune response to SE?

(d) Are heterologous spleen and thymus cells from rats capable of co-operation?

Previous reports, as discussed in various review articles (1,31, 32,33), have observed that thymus-deprived cultures could be stimulated to give an immune response by the addition of unidentified soluble mediators obtained from tissue culture supernatants. For this reason, supernatants from thymus cells and spleen cells were investigated for their ability to mediate an in vitro immune response to SE in nude spleen cell cultures.

Providing spleen cells cultured from nude mice failed to respond to SE, it seemed desirable to determine if a response could be obtained to an antigen that does not require the presence of T cells. This would be an indication of how a population of lymphocytes totally derived from the bone marrow react and respond to an antigen. The antigens chosen to investigate were LPS and SIII, though previous to this time no reports of an in vitro response to LPS had appeared in the literature.

The final phase of investigation was designed to determine if thymus lymphocytes bore the surface immunoglobulin IgM which was responsible for the cooperation between specific T and B cells. First, thymus cells were treated in vitro with various concentrations of serum specific for the heavy chain of IgMα (μ). Subsequently, an

in vivo system was used in which animals were suppressed with anti-μ from birth to six weeks of age when used as cell donors.

MATERIALS AND METHODS

Abbreviations

B cell - bone marrow-derived nonadherent lymphoid cell; BSS - balanced salt solution; C-LPS - heat killed *E. coli* cells; ^{60}Co - cobalt irradiation; LM - phenotypically normal littermates to nudes; LPS - lipopolysaccharide; LPS-SE - sheep erythrocytes coated with lipopolysaccharide; MEM - minimal essential medium, Eagle's; NRS - normal rabbit serum; PFC - plaque-forming cell; POL - polymerized flagellin; R - rad; SE - sheep erythrocytes; SIII - pneumopolysaccharide; T cell - thymus-derived nonadherent lymphoid cell.

Animals

Congenitally athymic mice (nu/nu), hereafter described as nude, and their phenotypically normal littermates (LM) (+/nu or +/+) were the offspring of heterozygous mice (+/nu) obtained by crossing nude males with Balb/c (+/+) females. The genetic background of the nude and LM mice was predominately Balb/c, and for this reason spleen cell cultures from Balb/c mice were used as a control for the culture system. LAF1 mice, bred from C571 females and A/He males, were used as a source of lymph node cells. Mice of the same age and sex were used in individual experiments and were 8 to 14 weeks old when used. Sasco white outbred rats 4 to 10 weeks of age were used as a source of spleen and thymus cells for some experiments.

Cell Cultures

Preparation of spleen cell suspensions and techniques of cell culture are essentially as described in detail by Mishell and Dutton (19). Briefly, mice were killed by cervical dislocation. The spleens and thymuses were removed aseptically and placed in a tissue culture grade plastic 60 X 15 mm Petri dish (Falcon Plastics) containing 10-15 ml of sterile balanced salt solution (BSS). Single cell suspensions from spleens and thymuses were then prepared by gentle teasing and sedimentation of tissue fragments and debris. Cells were suspended in Eagle's minimal essential medium (MEM) (Microbiological Associates, No. 12-126), supplemented with L-glutamine (1%, Microbiological Associates, No. 17-605F), nonessential amino acids (1%, Microbiological Associates, No. 13-114), sodium pyruvate (1%, Microbiological Associates, No. 13-115), and 5% fetal bovine serum (Reheis Co., Inc., Kankakee, Ill.) and containing 50 units per ml of penicillin and streptomycin. MEM supplemented as described was termed complete medium.

Cultures were established at 2.0-2.4 X 10^7 spleen cells per ml and thymus cells were plated at 2.5 or 5.0 X 10^7 per ml in addition to spleen cells. Cells in a total volume of 1 ml were maintained in 35 X 10 mm tissue culture grade plastic Petri dishes (Falcon Plastics) at 37°C on a continuously rocking platform in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2.

A nutritional mixture for daily feeding of the cultures was made as follows: 5 ml essential amino acids (50 X concentrated, Eagle, Microbiological Associates, No. 13-606), 25 ml nonessential amino acids (100 X concentrated, Eagle, Microbiological Associates, No. 13-114), 2.5 ml L-glutamine, 200mM (Microbiological Associates, No. 17-605F), 500 mg dextrose, and 35 ml MEM, Eagle, modified without $NaHCO_3$. The pH was adjusted to 7.2 with 1N NaOH and 7.5 ml of 7.5% $NaHCO_3$ added. The mixture was then sterilized by passage through washed Millipore cellulose filters (0.22μ pore size). Prior to use, fetal bovine serum was added to give a final volume of 1/3. Each culture dish was fed 0.09 ml of this nutritional mixture daily, after the first day.

Sheep Erythrocyte Antigen

Four blood samples, in Alsever's solution, from individual sheep were obtained from Colorado Serum Co. and tested in the culture system. Sheep No. 1786 gave the best results and erythrocytes from this animal were obtained every three weeks and used in all experiments. Cells were washed two times and 3×10^6 added to each culture dish (30 μl, or one drop from a pasteur pipette, of a 1% suspension of SE will give approximately this number).

Lipopolysaccharide Antigens

Soluble lipopolysaccharide (LPS), extracted by the phenol-water method (34) from *Escherichia coli* 0113, was kindly supplied by

Dr. J. A. Rudbach (University of Montana) and used in attempts to initiate an immune response to LPS. Soluble LPS and boiled soluble LPS were added to cultures in doses from 0.001 μg to 100 μg, in a 0.1 ml volume of complete medium.

To obtain whole cells as an antigen, *E. coli* 0113 was cultured in M-9 medium (35) containing 0.5% glucose. The cells were washed, suspended in a small amount of distilled water, and killed by heating in a boiling water bath for three hours. Prior to use in the system, 0.1 ml of this suspension was washed three times in BSS and diluted in complete medium (see Materials and Methods, Cell Cultures) to deliver 4.0×10^6 cells in the 30 μl added to each culture. This cellular antigen was designated C-LPS.

Pneumococcal Polysaccharide Type III Antigens

Soluble pneumococcal polysaccharide (SIII), prepared by a modification of the procedure of Felton, Kaffmann, and Stahl (36), and heat-killed pneumococcal cells were kindly supplied by Dr. P. J. Baker (National Institutes of Health, Bethesda, MD).

Thymus Grafting

Nude mice 4 to 6 weeks of age were thymus grafted by placing 4 to 8 thymuses from either littermates or Balb/c neonates (less than 5 days old) in the axillary region. At least two months were allowed

before using their spleens in cultures, and part of the animals were shown to demonstrate thymic function by rejecting heterografts. To avoid 'masking' of nonresponders, individual animals were used in the preparation of cell cultures.

Cell Separation Techniques

The cell separation techniques to obtain adherent and nonadherent cells were based on those described by Mosier (37) and explained in greater detail by Hartmann et al. (38). The cell separation techniques were used on either spleen (source of both adherent and nonadherent cells) or peritoneal cells (source of adherent cells).

Culture Supernatants

In attempts to determine if a humoral factor was released by antigen stimulated thymus or spleen cells, Balb/c thymus and spleen cells were cultured for 24 hours under normal culture conditions. The supernatant was harvested from these cells, with complete cell removal being obtained by centrifugation, and used to culture nude spleen cells. Since the culture medium was already one day old, nutritional mixture was added on day zero.

Neuraminidase

Neuraminidase from *Clostridium perfringens* (Nutritional Biologicals Corporation) was added to spleen cell cultures from nude mice

in attempts to obtain an immune response to SE. On day zero, 0.3 enzyme units were added to spleen cell cultures.

Irradiation

Thymus cells and thymus-derived spleen cells were treated with cobalt irradiation. Cells were harvested, washed in BSS, resuspended in either BSS or supplemented MEM, and irradiated while in an ice bath. To insure constant dosage, 15 ml glass centrifuge tubes containing no more than 4 ml of suspended cells were slanted to decrease depth and increase surface area and were agitated by shaking every 2 to 3 minutes during irradiation. Thymus-derived spleen cells were obtained by intravenously injecting lethally irradiated mice with 1.0 to 1.5 X 10^8 syngeneic thymus cells and 10^8 SE. Seven days later the spleens of these animals were harvested to obtain the "educated" thymus cells.

Anti-μ Serum

Pure rabbit anti-mouse IgM heavy chain (μ) and normal rabbit serum treated in the same manner (30) were obtained from Dr. Dean D. Manning.

Hemolytic Plaque Assay

The plaque forming cell (PFC) response of cultured cells was enumerated on day five by a slide modification (19) of the localized hemolysis-in-gel technique of Jerne. Agarose (Sigma Chemical Co.,

St. Louis, MO) from a single lot was used in all experiments. To detect PFC specific for LPS (14), sheep erythrocytes (SE) were coated with LPS (LPS-SE) (39) purified (34) from the same *E. coli* 0113 strain used as antigen. SE coated with SIII were used for plaquing and rabbit anti-mouse IgM antiserum was used to facilitate plaque development (41).

RESULTS

Selection of Sheep Erythrocytes

A previous observation by Mishell and Dutton (19) noted that erythrocytes from different sheep varied in their ability to stimulate an in vitro immune response. Both good and poor immune response stimulating erythrocytes can be found in different breeds of sheep, but a single animal is consistent. For this reason, the first experiment was to determine the ability of various SE to stimulate an in vitro immune response. Samples of blood from four individual sheep were obtained from Colorado Serum Company. Each sample was tested at the same time and under identical conditions. LAF1 mice were utilized as spleen cell donors since the hybrid animal normally gives a better immune response. In this experiment (Table I), SE from sheep 1786 were observed to stimulate the best in vitro immune response, and blood was obtained from this animal for all future experiments.

Comparison of Direct and Indirect Plaque-forming Cell Counts

The in vitro immune response is a primary response in that it is the first exposure to the heterologous erythrocyte antigen. The kenetics are similar to the in vivo response in most ways, but it seemed desirable to determine whether an indirect of IgG response was being stimulated to SE. Duplicate Jerne slides were prepared after culturing Balb/c and LM spleen cells and half were developed in the

Table I. Selection of sheep erythrocytes.[a]

Sheep Number[b]	PFC[c]	
	culture	10^6
Control[d]	48	10
1784	2400	488
1785	1500	334
1786	3000	644
1787	1950	407

a - A spleen pool for all cultures was prepared from 5 LAF1 mice.

b - Samples of blood were obtained from 4 different sheep (Colorado Serum Company) and 3 x 10^6 erythrocytes were added to each culture.

c - Number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

d - Background response in cultures without the addition of sheep erythrocytes (SE).

usual manner (direct) and half were facilitated with anti-IgG1,2. The response (Table II) showed that facilitation resulted in fewer plaque-forming cells. It thus appeared that anti-IgG had a suppressive effect on direct plaques and no conclusions could be made concerning IgG production in primary cell cultures.

Failure of Nude Spleen Cells to Respond to SE in vitro

Having demonstrated that the system was operative, that erythrocytes from the selected sheep initiated a good response, and that the direct PFC response was greater than the indirect PFC response, spleen cells from nude mice were cultured to determine if they were capable of responding in cell culture to SE. As seen in Table III, and as previously reported (42), nude spleen cells failed to respond to SE while Balb/c and LM spleen cells responded well. Attempts to obtain a response by increasing the cell concentration two-fold to 4.8 X 10^7 cells per culture were uniformly unsuccessful. The greater background and response to SE in these cultures could be attributed to the increased number of cells present.

It was observed during these first experiments with nude spleen cells, and in all additional experiments, that the number of cells recovered from nude cultures was consistently lower than from either LM or Balb/c cultures. For this reason, the PFC per culture response

Table II. Direct and indirect plaque comparison in primary cell cultures.

Cell Source[a]	Direct PFC[b] culture	Direct PFC[b] 10^6	Indirect PFC[c] culture	Indirect PFC[c] 10^6
Balb/c	1320	244	222	41
Littermate[d]	4800	678	2100	194

a - 2.0 x 10^7 spleen cells/ml/culture plus 3 x 10^6 SE.

b - Number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

c - Number of indirect plaque-forming cells (facilitated with anti-IgG1,2) per culture and per 10^6 nucleated cells recovered on day five.

d - Littermates to nudes.

Table III. Failure of nude spleen cells to respond to SE in vitro.

Cell Source[e]	-SE[a]; PFC per[b] culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] culture	+SE[a]; PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
Nude	4	2	7	3	12	30
Littermate	56	4	1955	284	5	13
Balb/c	84	24	2040	329	11	34

a - -SE, sheep erythrocytes not added to cultures; + SE, 3 x 10^6 sheep erythrocytes added to cultures.

b - Mean number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

c - Total number of experiments.

d - Total number of mice used in experiments.

e - 2.0-2.4 x 10^7 spleen cells/ml/culture.

converts to a larger PFC per 10^6 cells recovered response than with Balb/c or LM.

In vivo data reported by Taylor and Wortis (43) has shown that the PFC response of neonatally thymectomized mice increased with an increased immunizing dose of SE. Reed and Jutila (17) observed a similar phenomenon when large doses of SE were given to nudes. With increased immunizing doses of SE, the number of direct PFC in neonatally thymectomized mice and nudes increased, while the direct PFC response of normal animals decreased. Therefore, it was attempted to obtain an in vitro response in nude cultures by varying the antigenic dose of SE. In Table IV it is observed that a two-fold decrease to ten-fold increase in the number of SE had no effect on the response of nude spleen cell cultures. In comparison, decreasing the antigenic dose of SE two-fold had little effect on Balb/c or LM cultures, while high doses possibly inhibited or decreased the Balb/c PFC response (Table IV). From this data, a dose of 3 X 10^6 SE was selected for routine use.

Establishment of an Immune Response in Nude Spleen Cell Cultures with Balb/c and LM Thymus Cells

Having determined that nude spleen cell cultures failed to respond to SE, even with increased spleen cell and antigen concentration, the next question that was asked was whether the missing cell could be

Table IV. In vitro effect of various antigenic doses.

Cell Source[a]	No. of SE[b] per culture	PFC per[c] culture	PFC per[c] 10^6	No. of Expt.[d]	No. of Mice[e]
Nude	0	5	3	2	4
Nude	1.5×10^6	9	6	2	4
Nude	3.0×10^6	6	5	2	4
Nude	15.0×10^6	9	6	2	4
Nude	30.0×10^6	9	3	2	4
Littermate	0	12	3	2	4
Littermate	3.0×10^6	672	219	2	4
Littermate	15.0×10^6	540	223	2	4
Littermate	30.0×10^6	763	194	2	4
Balb/c	0	20	6	2	5
Balb/c	1.5×10^6	744	263	2	5
Balb/c	3.0×10^6	1026	306	2	5
Balb/c	15.0×10^6	906	231	2	5
Balb/c	30.0×10^6	564	150	2	5

a - 1.2×10^7 spleen cells/ml/culture.

b - Number of sheep erythrocytes added to each culture.

c - Mean number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

d - Number of experiments.

e - Total number of mice used in experiments.

Table V. Establishment of an immune response in nude spleen cell cultures with Balb/c and littermate thymus cells.

Cell Source	-SE[a]; PFC per[b]		+SE[a]; PFC per[b]		No. of	
	Culture	10^6	Culture	10^6	Expt.[c]	Mice[d]
Nude Spleen[e]	5	2	35	7	6	15
LM Spleen[e]	56	4	1955	284	5	13
Balb/c Spleen[e]	104	30	1968	320	4	19
LM Thymus[f]	0	0	0	0	2	6
Nude Spleen[e] + LM Thymus[f]	35	5	1820	115	2	6
LM Spleen[e] + LM Thymus[f]	217	7	2890	144	2	6
Balb/c Thymus	0	0	0	0	3	9
Nude Spleen[e] + Balb/c Thymus[f]	130	20	2533	130	5	13
Balb/c Spleen[e] + Balb/c Thymus[f]	282	20	2595	197	4	19

a - -SE, sheep erythrocytes not added to cultures; +SE, 3×10^6 sheep erythrocytes added to cultures.

b - Mean number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

c - Number of experiments.

d - Total number of animals used in experiments.

e - $2.0\text{-}2.4 \times 10^7$ cells/ml/culture.

f - 5.0×10^7 thymus cells/ml/culture.

replaced by directly adding thymus cells to nude spleen cell cultures. In experiments to establish an immune response in nude spleen cell cultures, the number of spleen cells cultured was held constant (2.4×10^7). To each such culture were added 5.0×10^7 littermate or Balb/c thymus cells. In preparing thymus cell suspensions, special care was taken to exclude the parathymic lymph nodes by injecting carbon black intraperitoneally 30 minutes prior to removal of the thymuses (44).

CBA thymus cells were used in a few experiments to establish an immune response in nude spleen cell cultures, but were not observed to be as efficient as Balb/c or LM thymus cells.

Establishment of an Immune Response in Nude Spleen Cell Cultures with Balb/c and LM Spleen Cells

The spleen is another source in which a large number of the cells present are derived from the thymus. In these experiments, Balb/c or littermate spleen cells were added in varying numbers to a constant number of spleen cells from nude animals. The same concentration of Balb/c spleen cells was cultured alone to insure that the Balb/c spleen cells were not the source of all the PFC in responding nude spleen cell cultures. The results in Table VI show that Balb/c spleen cells in low concentration do not respond in vitro. The addition of as few as 2.4×10^5 normal Balb/c spleen cells, however, resulted in establishing an immune response in nude spleen cell cultures. The addition of as few as

Table VI. Establishment with Balb/c spleen cells of an immune response in nude spleen cell cultures.

Spleen Cell Source	-SE[a]; PFC per[b] Culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] 10^6	No. of Expt.[c]	No. of Mice[d]
Nude[e]	5	2	35	7	6	15
Balb/c (2.4×10^7)	104	30	1968	320	4	19
Nude[e] + Balb/c (2.4×10^7)	161	18	3745	334	2	5
Balb/c (4.8×10^6)	0	0	12	38	1	4
Nude[e] + Balb/c (4.8×10^6)	175	38	5362	964	3	8
Balb/c (2.4×10^6)	0	0	0	0	1	4
Nude[e] + Balb/c (2.4×10^6)	287	69	4147	1427	3	9
Balb/c (2.4×10^5)	0	0	0	0	1	4
Nude[e] + Balb/c (2.4×10^5)	25	5	1783	610	3	9
Balb/c (2.4×10^4)	0	0	0	0	1	4
Nude[e] + Balb/c (2.4×10^4)	0	0	35	8	1	3

a-e - See Table V.

Table VII. Establishment with littermate spleen cells of an immune response in nude spleen cell cultures.

Spleen Cell Source	-SE[a]; PFC per[b]		+SE[a]; PFC per[b]		No. of	
	Culture	10^6	Culture	10^6	Expt.[c]	Mice[d]
Nude[e]	5	2	35	7	6	15
Littermate (2.4×10^7)	56	4	1955	284	5	13
Nude[e] + LM (2.4×10^7)	250	30	5903	586	3	8
Nude[e] + LM (4.8×10^6)	--	--	840	123	1	3
Nude[e] + LM (2.4×10^6)	--	--	2337	874	2	6
Nude[e] + LM (2.4×10^5)	--	--	259	71	1	3

a-e - See Table V.

2.4×10^5 Balb/c spleen cells to nude spleen cell cultures resulted in greater numbers of PFC per 10^6 than 2.4×10^7 Balb/c spleen cells alone (Table VI). Because of genetic variation among littermates, inbred Balb/c mice were used for most investigative studies.

In addition to Balb/c and littermate, CBA spleen cells were used in a few experiments. CBA spleen cells responded poorly and did not cooperate as efficiently as LM or Balb/c spleen cells with nude spleen cells to give an immune response to SE.

In Vitro Response of Spleen Cells from Thymus Grafted Nude Mice

Thymus and spleen cells are capable of establishing an in vitro culture response of nude spleen cells to SE. With this information, it seemed desirable to determine if nude animals grafted with intact thymus glands were reconstituted and able to respond in culture. Nude mice were grafted with either Balb/c or littermate thymuses by surgically implanting the glands in the axillary region. Two months or longer were allowed prior to using their spleens in culture, and part of these animals (N-1, N-2, and N-4) were shown to demonstrate thymic function by rejecting heterografts of either chicken or human skin. As the results show in Table VIII, spleen cell cultures from three of four thymus grafted nudes responded well to SE. It is interesting to note (Table VIII) that though the N-4 thymus grafted nude failed to respond, its spleen cells were capable of establishing an immune

Table VIII. Response of spleen cell cultures from thymus-grafted nude mice.

Cell Source	-SE[a]; PFC per[b]		+SE[a]; PFC per[b]	
	Culture	10^6	Culture	10^6
Nude[c]	7	3	84	24
N-1 (Thymus grafted)[d]	162	22	2400	360
N-2 (Thymus grafted)[e]	258	24	3420	279
N-3 (Thymus grafted)[f]	408	82	3120	778
N-4 (Thymus grafted)[g]	180	ND[h]	180	ND[h]
Nude[c] + N-4 spleen[i] (2.4×10^6)	ND[h]	ND[h]	2310	630

a - -SE, sheep erythrocytes not added to cultures; +SE, sheep erythrocytes added to cultures.

b - Number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

c - 2.4×10^7 normal nude spleen cells.

d - 2.4×10^7 spleen cells from thymus grafted nude; born 1-10-73; grafted with Balb/c thymuses 3-16-73; grafted with human skin 4-23-73; rejected; used in culture 6-14-73.

e - 2.4×10^7 spleen cells from thymus grafted nude; born 3-5-73; grafted with Balb/c thymuses 4-3-73; grafted with chicken skin 4-9-73, rejected; used in culture 6-14-73.

f - 2.4×10^7 spleen cells from thymus grafted nude; born 2-21-73; grafted with littermate thymuses 3-22-73; received littermate thymus cell i.p. 4-6-73; used in culture 6-21-73.

g - 2.4×10^7 spleen cells from thymus grafted nude; born 1-1-73; grafted with Balb/c thymuses 2-5-73; rejected chicken skin in 17 days; used in culture 6-21-73.

h - Not determined.

i - 2.4×10^7 normal nude spleen cells plus 2.4×10^6 spleen cells from N-4, a thymus grafted nude.

response to SE in normal nude spleen cell cultures. A normal level response was obtained by adding 2.4 X 10^6 N-4 cells to 2.4 X 10^7 normal nude spleen cells (Table VIII). The ability of spleen cells from N-1, N-2, and N-3 to establish an immune response in nude spleen cell cultures was not investigated.

Functional Adherent Cells Present in Nude Spleen

To determine if the antigen processing adherent cell reported by Mosier (37) to be required for an in vitro immune response to sheep erythrocytes was functional in the cell cultures from nude spleens, cells were separated on their ability to adhere to plastic and glass. Various combinations of adherent and nonadherent cells from nude and Balb/c spleens were tested to determine functional activity. In addition, adherent cells obtained from the peritoneal cavity of LM were added to normal nude spleen cells to determine if the adherent cell from this source was capable of reconstituting nude spleen cell cultures.

As seen in Table IX, nude spleen cell cultures failed to respond, while Balb/c spleen cell cultures responded well. Balb/c adherent and Balb/c nonadherent cells alone failed to respond, while in combination they gave low, but real responses. When this combination consisted of Balb/c adherent and nude nonadherent cells, no response was obtained (Table IX), but when the combination was reversed, Balb/c

Table IX. Adherent and nonadherent cell combinations.

Cells Cultured	-SE[a]; PFC per[b] Culture	10^6	+SE[a]; PFC per[b] Culture	10^6
Balb/c spleen[c]	60	9	1386	254
Nude spleen[c]	0	0	0	0
Balb/c adherent[d]	0	0	0	0
Balb/c nonadherent[e]	0	0	0	0
Balb/c adherent[d] + nonadherent[e]	0	0	210	151
Balb/c nonadherent[e] + Nude adherent[d]	0	0	147	75
Balb/c adherent[d] + Nude nonadherent[e]	0	0	0	0
Nude spleen[c] + Balb/c nonadherent[e]	252	100	6370	1250
Nude spleen[c] Littermate adherent[f]	0	0	0	0

a-c - See Table V.

d - Adherent cells obtained from the spleen, obtained by their ability to attach to plastic.

e - Nonadherent cells obtained from the spleen, obtained by removing adherent cells that attached to glass beads.

f - Adherent cells obtained from peritoneal cavity of littermates to to nudes, and obtained by their ability to attach to plastic (Adherent cells alone did not respond to SE in culture).

nonadherent and nude adherent cells, a response to SE was obtained. Additional evidence that the nude adherent cell population was functional and that the cell required for an immune response to SE was contained in the nonadherent cell population of Balb/c spleen, was obtained by adding approximately 1 X 10^5 Balb/c nonadherent cells to a normal population of nude spleen cells. The addition of this low number resulted in establishing a good response among nude spleen cells to SE (Table IX). The low response obtained with combinations of adherent and nonadherent cells is not unusual (37) and can mainly be attributed to the low numbers of cells cultured (19, Table VI).

Ability of C57Bl/Ks Lymph Node Cells to Establish an Immune Response in Nude Spleen Cell Cultures

Lymph node cells were harvested from C57Bl/Ks mice and added to nude spleen cell cultures to determine if allogeneic lymphoid cells could establish an immune response to SE. Two concentrations of lymph node cells were selected and used in two separate experiments. In the first experiment, 2.4 X 10^5 C57Bl/Ks lymph node cells were added to 2.4 X 10^7 nude spleen cells. The results in Table X show that a low response was obtained by adding this number of lymph node cells to the normal number of nude spleen cells. In contrast, the addition of this same number of lymph node cells to Balb/c spleen cells reduced the PFC response considerably. The response of nude spleen cell

Table X. Effect of low numbers of C57Bl/Ks lymph node cells on nude and Balb/c spleen cell cultures.

Cell Source	-SE[a]; PFC per[b]		+SE[a]; PFC per[b]	
	Culture	10^6	Culture	10^6
Nude[c]	7	1	7	1
Nude[c] + C57Bl/Ks lymph node cells (2.4 x 10^5)	7	1	357	42
Balb/c[c]	21	3	1320	220
Balb/c[c] + C57Bl/Ks lymph node cells (2.4 x 10^5)	21	4	455	88

a-c - See Table V.

Table XI. Effect of increased numbers of C57Bl/Ks lymph node cells on nude and Balb/c spleen cell cultures.

Cell Source	-SE[a]; PFC per[b]		+SE[a]; PFC per[b]	
	Culture	10^6	Culture	10^6
Nude[c]	36	12	36	12
Nude[c] + C57Bl/Ks lymph node cells (2.4 x 10^6)	46	8	5320	752
Balb/c[c]	21	3	1734	213
Balb/c[c] + C57Bl/Ks lymph node cells (2.4 x 10^6)	21	4	2030	255

a-b - See Table V.

cultures obtained by adding 2.4 X 10^5 C57Bl/Ks lymph node cells was much lower than that observed with the same number of Balb/c spleen cells (Table VI), but was comparable to the response obtained using the same number of littermate spleen cells (Table VII).

To determine if the low response in nude spleen cell cultures with C57Bl/Ks lymph node cells was due to insufficient numbers of co-operating thymus-derived cells and if C57Bl/Ks lymph node cells truly suppressed the response of Balb/c cultures, the experiment was repeated using an increased number of lymph node cells. In this experiment, 2.4 X 10^6 lymph node cells were added to nude and Balb/c spleen cell cultures. As seen in Table XI, this established a strong SE response in nude spleen cell cultures and rather than having a suppressive effect on Balb/c cultures, this addition slightly enhanced the SE response both in terms of PFC per culture and per 10^6 recovered cells. These data indicated that the low response obtained with 2.4 X 10^5 lymph node cells was due to insufficient numbers of cooperating cells and the response obtained with 2.4 X 10^6 lymph node cells (Table XI) compares well to the response obtained in nude spleen cell cultures with an equal number of 2.4 X 10^6 Balb/c spleen cells (Table VI). The data also indicated that the suppressive effect observed in Balb/c spleen cell cultures with the lower number of C57Bl/Ks lymph node cells was probably an artifact because a larger number of lymph node cells failed to exhibit this suppressive effect.

Attempts to Establish an Immune Response in Nude Spleen Cell Cultures with Heterologous Thymus and Spleen Cells

With the evidence that the response in nude spleen cell cultures can probably occur across H-2 histocompatibility loci (CBA, $H\text{-}2^k$; Balb/c, $H\text{-}2^d$), it was decided to attempt to obtain a response to SE with heterologous (rat) lymphoid cells. The procedure was basically the same as used for establishing an immune response in nude spleen cell cultures with Balb/c or littermate spleen and thymus cells. Because nude spleen cells failed to respond to SE in vitro, controls for the experiments were the establishment of a response in nude spleen cell cultures with Balb/c thymus and spleen cells. In addition, Balb/c liver was added to cultures as a control to ensure that cell cooperation with nude spleen cells required lymphoid cells.

Table XII shows that rat spleen and thymus cells were ineffective in establishing an immune response in nude spleen cell cultures. Normal results were obtained with nude and Balb/c cultures, and with their various cell combinations (Table XII and Tables V and VI). Rat spleen cells failed to respond to SE in cell cultures, and the addition of rat or Balb/c thymus cells did not overcome unresponsiveness (Table XII). Rat thymus cells, upon addition to normal Balb/c spleen cell cultures, were observed to totally inhibit the immune response and background response to SE (Table XII). Liver cells also suppressed

Table XII. Attempts to establish an immune response in nude spleen cell cultures with heterologous (rat) thymus and spleen cells.

Cell Source	-SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] Culture	Expt.[c]	Animals[d]
Nude spleen[e]	0	0	2	5
Nude spleen[e] + Balb/c thymus[f]	48	1320	2	5
Nude spleen[e] + rat thymus	3	36	2	5
Nude spleen[e] + Balb/c spleen (2.4×10^6)	--	1120	2	5
Nude spleen[e] + rat spleen (2.4×10^6)	0	14	2	5
Nude spleen[e] + Balb/c liver[g] (2.3×10^7)	0	0	1	3
Balb/c spleen[e]	186	1956	2	8
Balb/c thymus[f]	0	0	2	8
Balb/c spleen[e] + Balb/c thymus[f]	315	3630	2	8
Balb/c spleen[e] + rat thymus[f]	0	0	2	8
Balb/c spleen[e] + rat spleen (2.4×10^6)	--	1050	1	5
Balb/c spleen[e] + Balb/c liver[g] (2.3×10^7)	0	0	1	3
Rat spleen[e]	0	0	2	3
Rat spleen[e] + rat thymus[f]	0	0	2	3
Rat spleen[e] + Balb/c thymus[f]	0	0	2	3

a-f - See Table V.

g - Liver cells were added as a non-lymphoid control to insure that non-specific factors were not involved.

the response to SE in Balb/c spleen cell cultures, but this was not unexpected due to the nature of liver cells (high lipid content, etc.).

Effect of Neuraminidase on Spleen Cell Cultures

It was postulated that neuraminidase from *Clostridium perfringens* might stimulate a response to SE in nude spleen cell cultures and increase the response in Balb/c spleen cell cultures. To determine this, 0.3 enzyme units were added to nude and Balb/c spleen cell cultures on day zero. The results in Table XIII indicate that the enzyme did not stimulate nude spleen cells to respond to SE and totally abolished the SE response in Balb/c spleen cell cultures. As controls for the system, Balb/c spleen cells gave a normal response to SE, and nude spleen cell cultures responded with the addition of Balb/c thymus cells (Table XIII).

Attempts to Establish an Immune Response with Supernatants

With data demonstrating that an immune response could be established in nude spleen cell cultures with Balb/c, CBA, LM spleen or thymus cells, or with C57Bl/Ks lymph node cells, it was decided to investigate whether supernatants from thymus and spleen cells were capable of establishing a response. Supernatants obtained from 24 hour cultures of Balb/c spleen and thymus cells in which SE had been present were

Table XIII. Effect of neuraminidase on spleen cell cultures.

Cell Source	-SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] Culture
Nude spleen[c]	0	0
Nude spleen[c] + Balb/c thymus[d]	120	1560
Nude spleen[c] + neuraminidase[e]	0	0
Balb/c spleen[c]	12	420
Balb/c spleen[c] + neuraminidase[e]	0	0

a-b - See Table V.

c - 2.0-2.4 x 10^7 cells/ml/culture.

d - 5.0 x 10^7 cells/ml/culture.

e - Neuraminidase (0.3 enzyme units) from _Clostridium perfringens_ added to each culture.

used to culture freshly prepared nude and Balb/c spleen cells (Since the culture medium was already 24 hours old, nutritional mixture was added on day zero). In addition, Balb/c spleen and thymus cells were exposed to 1200 R of ^{60}Co irradiation and then cultured for 24 hours to obtain supernatants.

The results in Table XIV show that nude spleen cells were not stimulated to respond to SE with any of the supernatants. Neither thymus or spleen supernatants undiluted or diluted 1:10 in complete culture medium, or supernatants from irradiated thymus or spleen cells, were able to establish an immune response in nude spleen cell cultures. The controls, which included normal Balb/c thymus and spleen cells added to nude spleen cells, responded in a normal manner (Table XIV and Tables V and VI).

Extended conclusions are not possible from these experiments because supernatants were often suppressive to Balb/c spleen cultures (Table XIV). In both experiments (Table XIV), undiluted supernatants from thymus cells were totally suppressive, with a normal response being obtained by diluting the thymus cell supernatant ten-fold. In one experiment, supernatant from Balb/c spleen cells was observed to have a suppressive effect, but a normal response was obtained in the following experiment with undiluted and diluted spleen cell supernatants (Table XIV). Supernatants from irradiated thymus and spleen cells were

Table XIV. Attempts to establish an immune response in nude spleen cell cultures with supernatants prepared from Balb/c spleen and thymus cells.

Cell & supernatant combinations	+SE[a]; PFC per[b] culture	
	Expt. #1	Expt. #2
Nude[c]	0	0
Nude[c] + thymus cells[d]	1050	1680
Nude[c] + thymus supernatant[e]	0	0
Nude[c] + thymus supernatant 1:10[f]	--	0
Nude[c] + irradiated thymus supernatant[g]	0	--
Nude[c] + spleen cells (2.4×10^6)	1920	1190
Nude[c] + spleen supernatant[e]	0	0
Nude[c] + spleen supernatant 1:10[f]	--	0
Nude[c] + irradiated spleen supernatant[g]	0	--
Balb/c[c]	1920	1092
Balb/c[c] + thymus cells[d]	1320	1860
Balb/c[c] + thymus supernatant[e]	0	0
Balb/c[c] + thymus supernatant 1:10[f]	--	1260

Table XIV (continued).

Cell & supernatant combinations	+SE[a]; PFC per[b] culture Expt. #1	Expt. #2
Balb/c[c] + irradiated thymus supernatant[g]	0	--
Balb/c[c] + spleen supernatant[e]	0	1200
Balb/c[c] + spleen supernatant 1:10[f]	--	1120
Balb/c[c] + irradiated spleen supernatant[g]	0	--

a - +SE, sheep erythrocytes added to cultures (background to SE was less than 5% in all combinations checked).

b - Plaque-forming cell response per culture, assayed on day five.

c - 2.0-2.4 x 10^7 Balb/c or nude spleen cells/ml/culture.

d - 5.0 x 10^7 Balb/c or nude thymus cells/ml/culture.

e - Spleen cells cultured in 24 hour Balb/c thymus or spleen supernatant in which SE had been present.

f - Supernatants, prepared as described in footnote 'e', diluted 1:10 and then used to culture spleen cells.

g - As in footnote 'e' above, except that thymus and spleen cells were irradiated (1200r ^{60}Co) prior to 24 hour incubation to obtain supernatants.

also observed to totally suppress the immune response when used to culture Balb/c spleen cells.

Irradiation Resistance of Thymus Cells

Following the observation that thymus and spleen cell supernatants did not establish an immune response in nude spleen cell cultures, it was decided to approach the question of the role of the cooperating thymus cells (or thymus-derived cells) in another way. The mechanism used was ^{60}Co irradiation. The first experiment was to determine if the cooperating effect that occurs between Balb/c thymus cells and nude spleen cells was radiosensitive. Thymus cells were removed and suspended in complete medium. Half was subjected to 1200 R as described in Materials and Methods and half was maintained under similar conditions without irradiation. This preliminary experiment, as shown in Table XV, indicated that 1200 R of ^{60}Co irradiation inhibited the capacity of Balb/c thymus cells to establish an immune response to SE in nude spleen cell cultures. It was also observed (Table XV) that thymus cells receiving 1200 R totally suppressed the response of Balb/c spleen cells in vitro.

In subsequent experiments, the dosage of irradiation was varied to determine the sensitivity of thymus cells. The data in Table XVI indicated that low doses of irradiation inhibit the cooperating capacity

Table XV. Irradiation of thymus cells. I. High dose effect on normal thymus cells.

Cell combinations	+SE[a]; PFC per[b] Culture
Nude[c]	0
Nude[c] + thymus[d]	1560
Nude[c] + thymus 1200r[e]	0
Balb/c[c]	490
Balb/c[c] + thymus 1200r[e]	0

a - +SE, sheep erythrocytes added to cultures; background in unimmunized cultures was less that 10% of the observed response.

b - Plaque-forming cells per culture on day five.

c - 2.4×10^7 spleen cells/ml/culture.

d - 5.0×10^7 Balb/c thymus cells/ml/culture.

e - 5.0×10^7 Balb/c thymus cells which had received 1200 R irradiation from a ^{60}Co source.

of thymus cells. A dose as low as 100 R reduced the response 54-79 per cent, and 400-800 R totally inhibited Balb/c thymus cells from establishing an immune response to SE in nude spleen cell cultures. A different effect was observed when irradiated thymus cells were added to Balb/c spleen cell cultures (Table XVI). A low dose of irradiation (100 R) suppressed the response to less than 10 per cent of the control level which was Balb/c spleen cells combined with thymus cells which had not been irradiated. With higher doses of irradiation, 200 and 400 R, the response was near that of the unirradiated controls (Table XVI).

Irradiation Resistance of Educated Thymus Cells

The experiments with irradiated thymus cells indicated that the thymus cell was highly sensitive to ^{60}Co irradiation. Since in the normal intact animal the T cells that are involved in the immune response are those found mainly in the spleen, lymph nodes, etc., it seemed likely that maturation and resulting irradiation resistance could occur after cells leave the thymus. To artificially create maturation of thymus cells, a system utilizing lethally irradiated (900 R) animals was used. Balb/c mice were lethally irradiated, injected intravenously with 1.0-1.5 X 10^8 syngeneic thymus cells and 10^8 SE, and one week later their spleens harvested for a source of

Table XVI. Irradiation of thymus cells. II. Effect of various doses.

Cell Combinations	±SE[a]; PFC per culture[b]	
	Expt. 2	Expt. 3
Nude[c]	7	7
Nude[c] + thymus[d]	560	420
Nude[c] + thymus 100r[e]	116	192
Nude[c] + thymus 200r[e]	49	90
Nude[c] + thymus 400r[e]	0	12
Nude[c] + thymus 800r[e]	0	--
Balb/c[c]		2100
Balb/c[c] + thymus[d]		833
Balb/c[c] + thymus 100r[e]		60
Balb/c + thymus 200r[e]		660
Balb/c + thymus 400r[e]		768

a-d - See Table XV.

e - 5 x 10^7 Balb/c thymus cells/ml/culture, irradiated with the number of rads indicated using a ^{60}Co source.

educated thymus cells. The theory was that thymus cells capable of reacting to SE would contact the antigen in the lethally irradiated animal and be stimulated to divide, creating an enriched thymus-derived population that was reactive to SE. As a control on the ability of 900 R to destroy immunocompetent cells in vivo, lethally irradiated mice were injected intravenously with 10^8 SE. Educated thymus cells were irradiated in vitro as described in Materials and Methods and used in attempts to establish an immune response in nude spleen cell cultures. In the same experiment it was also desirable to determine if low dose irradiation of educated thymus cells was suppressive to Balb/c spleen cell cultures, as was the low dose irradiation of T cells.

The data in Table XVII indicate that educated thymus cells remain sensitive to irradiation, though probably not as sensitive as normal thymus cells. A dose of 400 R was observed to suppress the activity of educated thymus cells to less than 70 per cent (Table XVII) compared to an almost total suppression obtained with treating normal thymus cells with the same dose (Table XVI).

The phenomenon of low irradiation dose suppression was again observed in the experiments in which educated thymus cells were added to Balb/c spleen cell cultures (Table XVII). One problem in interpreting the results shown in Table XVII is that in one of the two experiments, educated thymus cells which were not irradiated had a

Table XVII. Irradiation of thymus cells. III. Effect of various doses on educated thymus cells.

Cell Source	+SE[a] Expt. 1 PFC per culture[b]	+SE[a] Expt. 2 PFC/culture[b]	+SE[a] Expt. 2 PFC/10^6
Nude[c]		56	47
Nude[c] + ETC[d]		375	125
Nude[c] + ETC 100r[e]		345	125
Nude[c] + ETC 200r[e]		207	84
Nude[c] + ETC 400r[e]		111	58
ETC[d]	0	0	0
Balb/c[c]	3240	762	192
Balb/c[c] + ETC[d]	1246	243	55
Balb/c[c] + ETC 100r[e]	186	207	39
Balb/c[c] + ETC 200r[e]	834	1155	247
Balb/c[c] + ETC 400r[e]	1392	1028	232
Irradiation Control[f]	0		
Balb/c[c] + Irradiation Control[f]	1500		

a-c - See Table V.

d - Educated thymus cells, obtained by injecting lethally irradiated Balb/c mice with syngeneic thymus cells (1.0-1.5 x 10^8) and 10^8 SE, and harvesting spleens from these animals; four cultures prepared from each spleen giving 1.0-1.1 x 10^7 cells.

e - Educated thymus cells obtained as explained in footnote 'd' (above) and irradiated with the dose indicated.

f - Spleen cells obtained from a lethally irradiated animal given 10^8 SE; four cultures prepared from each spleen.

suppressive effect on Balb/c spleen cell cultures. Even so, 100 R of irradiation was even more suppressive and enhanced responses were obtained with 200 and 400 R.

The results of controls were essentially as expected. Spleen cells from lethally irradiated mice injected with SE only did not respond in culture and had no dramatic effect on the response of Balb/c or nude spleen cell cultures. Educated thymus cells also failed to give a response to SE in cell culture. These data indicated that 900 R totally destroyed the cells that could produce antibody to SE.

Thymus Independent Antigens

Evidence to this point indicated that the nude mouse possessed a functional macrophage or adherent cell and that the cell required to establish an in vitro immune response to SE was a nonadherent thymus to thymus-derived cell that was radiosensitive. Evidence for a normal bone marrow-derived population of cells was thus of an indirect nature, since the addition of cells was required to obtain an in vitro response to SE. To obtain a direct answer to the capacity of a pure bone marrow-derived cell population to respond to cell cultures, alleged thymus-independent antigens were investigated. Pneumococcal polysaccharide type III (SIII) had previously been reported (23) to give a low response in normal mouse spleen cell cultures. Attempts were made to obtain an immune response to this antigen in Balb/c and nude spleen

cell cultures with the reported dosage of 20 μg per ml (23) and with other doses ranging from 100 μg per ml per culture to 0.0001 μg per ml per culture. In addition, heat-killed pneumococcal cells were added to cultures to give the equivalent of 1.0, 0.1, 0.01, 0.001, and 0.0001 μg of SIII per ml per culture. All results were negative, and it became apparent that an immune response to this antigen was not to be readily obtained in cell cultures.

The thymus independent antigen lipopolysaccharide (LPS), or endotoxin, was then investigated as to its capacity to induce an immune response in vitro. Since the majority of the research investigating LPS had been performed with the soluble preparation of the compound, the experiments attempted in vitro were with the soluble form of LPS also. In vivo dose response data to LPS (45) had shown that 10 μg resulted in an optimal response, and that the peak response was obtained on day 4. With this information, doses of 100, 10, 1.0, 0.1, 0.01, 0.001, and 0.0001 μg were added to 1 ml cultures, and cultures were assayed for an immune response to LPS by plaquing on SE coated with LPS (LPS-SE). Again, negative results were obtained though it was noted that doses of 10 and 100 μg were stimulatory to cells and increased the number of harvested cells obtained on the day of assay. This was observed by comparing cell counts at these dosages to cultures that did not receive antigen, received SE, or received lower doses of LPS. The

number of recovered cells with 100 μg of LPS added to cultures was generally 40-60 per cent greater than control cultures with SE or no antigen. This observed increase in cell numbers with high doses of LPS was more pronounced in nude spleen cell cultures than in Balb/c cultures.

When negative results were obtained with soluble, phenol-water extracted LPS, it was decided to use LPS which had been boiled for three hours in a water bath. Endotoxin prepared in this manner has been found to attach more readily to cells and is used to coat SE for immunological assay (39). Boiled LPS was used in the same concentrations as previously used for LPS (100 to 0.0001 μg) and no response was obtained. Boiled LPS was not observed to have a stimulatory effect on spleen cell populations.

In a last attempt to obtain an immune response to LPS in vitro, E. coli 0113 cells were heat-killed (see Materials and Methods for preparation) and added to cultures. This is the same strain from which the soluble LPS had been purified, and to distinguish this antigen from soluble LPS it was termed C-LPS. C-LPS was added to cultures in doses from 4 X 10^5 cells per culture to 4 X 10^9 cells per culture. A response to LPS was obtained by adding 2 X 10^6 to 4 X 10^7 heat-killed E. coli cells, and additional investigation determined that the best response was obtained on day five of culture using 4 X 10^6 heat-killed cells

from the C-LPS preparation. A single batch of C-LPS antigen was prepared and used in all experiments.

As can be seen in Table XVIII, and as previously reported (46), nude spleen cell cultures did not respond to SE, but did respond to C-LPS. LM and Balb/c spleen cell cultures responded to both SE and C-LPS. It was observed that upon addition of C-LPS, the background to SE generally increased.

In comparing the responses to C-LPS, it was found that the per culture PFC response of nudes was lower than that of either LM or Balb/c cultures. This apparent discrepancy was due to lower recovery of cells from nude cultures.

Having obtained an in vitro immune response to LPS by using the whole killed cell, it seemed logical to ask if LPS bound to SE would, when added to nude spleen cell cultures, establish an immune response to SE. SE were conjugated with LPS in the same manner as for the plaque assay and added to nude spleen cell cultures. LPS-SE were added in various increasing concentrations, since previous data indicated that only small amounts of LPS bound to SE in the conjugating procedure (47). The normal SE dose as an antigen in the in vitro system is 3×10^6 cells. This number of LPS-SE was added, as were 3×10^7, 6×10^7, and 1.2×10^8 LPS-SE, to nude spleen cell cultures. In no case was the SE specific PFC response to LPS-SE greater than that observed with 3×10^6 SE alone.

Table XVIII. In vitro immune response to lipopolysaccharide.

Cell Source	Antigen	PFC on SE[a] Culture	PFC on SE[a] 10^6	PFC on LPS-SE[b] Culture	PFC on LPS-SE[b] 10^6	No. of Expt.[c]
Nude	---	4	1	4[e]	1[e]	5 (12)
LM[d]	---	28	4	21[e]	3[e]	1 (3)
Balb/c	---	90	16	66[e]	11[e]	7 (27)
Nude	SE	6	1	--	--	5 (12)
LM	SE	1800	273	--	--	1 (3)
Balb/c	SE	1741	304	--	--	7 (27)
Nude	E. coli[f]	11	3	462[g]	130[g]	5 (12)
LM	E. coli	162	29	792[g]	140[g]	1 (3)
Balb/c	E. coli	48	9	691[g]	155[g]	7 (27)

a - Mean number of direct plaque-forming cells (PFC) on sheep erythrocytes (SE) expressed per culture and per 10^6 cells recovered on day five.

b - Mean number of PFC on lipopolysaccharide-coated SE (LPS-SE) expressed per culture and per 10^6 cells recovered on day five.

c - Total number of experiments (total number of mice used in experiments).

d - Littermates of nude mice.

e - Background PFC to SE not subtracted.

f - Heat killed E. coli 0113 cells, 4 x 10^6 per culture.

g - Background PFC response to SE subtracted.

In Vitro Immunosuppression with Heterologous Anti-μ Antiserum

Having determined that nude spleen cells required a cooperating thymus or thymus-derived cell to respond to SE and that nude spleen cells have a functional bone marrow-derived cell population as shown by their ability to respond in cultures to an antigen that does not require the participation of T cells, a system in which to study the target cell of anti-μ suppression became evident. Previous studies by Manning and Jutila (30), utilizing nude mice and LPS as the thymus-independent antigen, indicated that the bone marrow-derived cell was suppressed by anti-μ serum. Cell transfer studies using suppressed animals and lethally irradiated recipients were totally inconclusive as to the cell affected by anti-μ suppression (30).

The first question was whether anti-μ would suppress the in vitro immune response. This was determined by adding 0.05 ml of various dilutions of anti-μ serum to 1 ml cultures of Balb/c spleen cells. As a control, normal rabbit serum (NRS), which had been treated by the same protocol as the anti-μ serum, was added in the same dilutions to other cultures.

As seen in Table XIX, normal rabbit serum at dilutions of 1:200, 1:400, and 1:1000 had little effect on the SE response of Balb/c spleen cell cultures. In contrast, the 1:400 dilution of anti-μ serum suppressed the response to SE to less than 10 per cent of normal and the

Table XIX. Immunosuppressive activity of anti-μ serum. I. Determination of in vitro activity.

Cell Treatment[a]	+SE[b]; PFC per[c] Culture	+SE[b]; PFC per[c] 10^6	No. of Expt.[d]	No. of Mice[e]
----	1939	238	3	8
anti-μ 1:200[f]	0	0	6	17
anti-μ 1:400[f]	138	17	3	8
anti-μ 1:1000[f]	1116	190	2	6
anti-μ 1:2000[f]	2960	405	1	3
NRS 1:200[g]	1860	208	3	8
NRS 1:400[g]	1920	240	3	8
NRS 1:1000[g]	2040	254	2	6
NRS 1:2000[g]	3100	364	1	3

a - 2.4×10^7 Balb/c spleen cells/ml/culture.

b - +SE, sheep erythrocytes added to cultures; background in all cases observed was less than 10% of response.

c - Mean number of plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

d - Number of experiments.

e - Total number of animals used in experiments.

f - Anti-μ serum diluted to give final concentration indicated.

g - Normal rabbit serum diluted to give final concentration indicated.

1:200 dilutions totally suppressed the response of Balb/c spleen cell cultures to SE. It was also observed that 1:2000 dilutions of NRS and anti-μ serum had a stimulatory effect on Balb/c cultures. Since the 1:200 dilution of anti-μ serum was totally suppressive, this dilution was selected for future studies.

Since Balb/c spleen cells contain both B and T cells, the data in Table XIX do not indicate the target cell for anti-μ suppression. An attempt to determine the target cell was performed by utilizing nude mice as a source of B cells and Balb/c thymus as a source of thymus cells. In two experiments, spleen and thymus cells were suspended to normal culture concentrations in BSS and then treated with NRS, anti-μ serum, or left untreated. Spleen and thymus cells were treated with 1:10, 1:100, or 1:200 dilutions of NRS or anti-μ serum for four hours at 4°C. Every 15 minutes during the treatment time tubes were shaken to ensure that cells remained suspension. Following the four hour treatment period, the cells were washed three times in BSS, and then suspended in complete MEM. Various combinations of normal and treated cells were combined to determine if suppression had occurred. The cell combinations and results of two experiments are seen in Table XX. The results of the two experiments are quite similar, but differ in that sera used for treatment were diluted 1:100 and 1:200 in the first experiment and 1:10 and 1:100 in the second.

Table XX. Immunosuppressive activity of anti-μ serum. II. In vitro pretreatment of cells.

Cell Source and Treatment	+SE[a]; PFC per culture[b]	
	Expt. 1	Expt. 2
Nude[c]	12	7
Nude[c] + thymus[d]	282	276
Nude[c] + thymus-NRS 1:10[e]	--	228
Nude[c] + thymus-anti-μ 1:10[f]	--	282
Nude[c] + thymus-NRS 1:100[e]	364	318
Nude[c] + thymus-anti-μ 1:100[f]	492	426
Nude[c] + thymus-NRS 1:200[e]	210	--
Nude[c] + thymus-anti-μ 1:200[f]	396	--
Nude[c] --anti-μ 1:10[g] + thymus[d]	--	0
Nude[c] --anti-μ 1:100[g] + thymus[d]	392	264
Nude[c] --anti-μ 1:200[g] + thymus[d]	504	--
Nude[c] + thymus-anti-μ 1:10[f] + Balb/c spleen[h]	--	1560
Nude[c] + thymus-anti-μ 1:100[f] + Balb/c spleen[h]	483	1020
Nude[c] + thymus-anti-μ 1:200[f] + Balb/c spleen[h]	1106	--
Balb/c[c]	2040	1200
Balb/c[c] + thymus[d]	1260	3780
Balb/c[c] + thymus-NRS 1:10[e]	--	3480
Balb/c[c] + thymus-anti-μ 1:10[f]	--	3000
Balb/c[c] + thymus-NRS 1:100[e]	1484	4320
Balb/c[c] + thymus-anti-μ 1:100[f]	1200	4320
Balb/c[c] + thymus-NRS 1:200[e]	1400	--
Balb/c[c] + thymus-anti-μ 1:200[f]	2160	--
Balb/c[c] --anti-μ 1:10[g]	--	0

Table XX (continued).

Cell Source and Treatment	+SE[a]; PFC per culture[b] Expt. 1	Expt. 2
Balb/c[c]--anti-μ 1:100[g]	0	12
Balb/c[c]--anti-μ 1:200[g]	0	--
Balb/c[c] + NRS 1:200[i]	1920	1380
Balb/c[c] + anti-μ 1:200[j]	0	0

a - +SE, sheep erythrocytes add to cultures; background to SE was less than 10% of response except with nude spleen cells where the background and response were equal.

b - Plaque-forming cells per culture.

c - 2.4×10^7 spleen cells/ml/culture.

d - 5.0×10^7 Balb/c thymus cells/ml/culture.

e - Balb/c thymus cells pretreated for 4 hr with normal rabbit serum at the indicated dilution.

f - Balb/c thymus cells pretreated for 4 hr with anti-μ serum at the indicated dilution.

g - Spleen cells pretreated for 4 hr with anti-μ serum at the indicated dilution.

h - 2.4×10^5 Balb/c spleen cells added to culture.

i - Normal rabbit serum added to culture to give the final concentration indicated.

j - Anti-μ serum added to culture to give the final concentration indicated.

The data in Table XX showed that NRS treatment in all concentrations had little or no effect on the response of cultures. The response of nude cultures reconstituted with NRS-treated thymus cells in any of the three dilutions employed gave responses similar to untreated thymus cells. It was also observed that the treatment of thymus cells with anti-μ serum had no effect on their ability to reconstitute nude cultures. Thymus cells, NRS or anti-μ treated, had little effect on Balb/c spleen cultures, with the exception that thymus cells treated with a 1:200 dilution of anti-μ enhanced the response of Balb/c spleen cells to SE.

The treatment of spleen cells with anti-μ did have a suppressive effect. When nude spleen cells were treated for four hours with a 1:10 dilution of anti-μ and normal thymus cells added, no response occurred to SE. When nude spleen cells were treated with 1:100 and 1:200 dilutions of anti-μ, suppression was not observed to occur. When Balb/c spleen cells were treated with anti-μ for four hours, suppression of the immune response to SE was essentially total. Additional controls for these experiments included Balb/c spleen cultures to which NRS 1:200 and anti-μ 1:200 were added to show that the anti-μ serum was suppressive, while NRS was not. A final set of controls was used in these experiments and would have been informative and necessary if anti-μ treated thymus cells had been suppressive in nude spleen cultures. If anti-μ treated thymus cells had failed to reconstitute nude

spleen cultures, it would have been necessary to rule out the possibility of carry over anti-μ that could affect B cells. For this reason, 2.4 X 10^5 normal Balb/c spleen cells were added to nude spleen cultures in which anti-μ treated thymus cells were present. If thymus cells had been suppressed, then the addition of the small number of spleen cells would have reconstituted the cultures, but if carry over anti-μ was the mechanism, then no reconstitution would occur because the Balb/c spleen cells would have been suppressed at the same time.

The conclusions from the above experiments were that B cells could be suppressed by anti-μ in vitro, but that the treatment of thymus cells with anti-μ under the conditions described above did not affect their ability to cooperate with B cells. The question of whether thymus cells were at any time suppressed or can be suppressed under other circumstances by anti-μ still remained unanswered.

To approach the question with a population of thymus cells that had been treated with anti-μ for a longer period of time, neonatal Balb/c mice were injected with NRS or anti-μ serum within the first 24 hours after birth. Heavy suppression continued during the following five weeks with the mice receiving serum injection every other day. At 38 days of age, having been on continuous NRS or anti-μ serum treatment, the thymuses and spleens were removed and cell cultures prepared from them. Serum was collected from these same animals, and the anti-

treated mice were shown to have a high level of circulating anti-μ activity. This high level of circulating rabbit anti-μ indicated that an excess of anti-μ was being injected, and that any cell having μ chains on its surface and being in contact with the circulatory system should have reacted with anti-μ.

The data in Table XXI showed that thymus cells from NRS and anti-μ serum treated mice were equally capable of establishing an immune response to SE in nude spleen cell cultures. An immune response was also obtained in cultures containing spleen cells from the NRS treated animals and thymus cells from the anti- treated animals. This showed that thymus cells from anti-μ treated animals did not have a suppressive effect on spleen cell cultures. The data in Tabls XXI also showed that the spleen cell cultures from the anti-μ treated animals were totally suppressed. A response was not obtained in cultures prepared from the spleens of anti-μ treated animals upon the addition of thymus cells from anti-μ treated animals nor with the addition of thymus cells from NRS treated animals (The PFC per culture response is probably of greater significance in Table XXI, for due to the large number of thymus cells added, the number of recovered cells was high, resulting in a low PFC response per 10^6 recovered cells).

The results from the in vitro and in vivo treatment of thymus cells and B cells with anti-μ serum strongly indicate that the target

Table XXI. Immunosuppressive activity of anti-μ serum. III. In vivo treatment of cells.

Cell Combinations	+SE[a]; PFC per[b] Culture	10^6
Nude spleen[c]	7	3
Nude spleen[c] + NRS thymus[d]	1356	71
Nude spleen[c] + anti-μ thymus[e]	1299	62
NRS spleen[f] + NRS thymus[d]	96	5
NRS spleen[f] + anti-μ thymus[e]	1134	52
Anti-μ spleen[g] + NRS thymus[d]	0	0
Anti-μ spleen[g] + anti-μ thymus[e]	0	0

a - +SE, sheep erythrocytes added to cultures.

b - Plaque-forming cells per culture or per 10^6 nucleated cells recovered on day five.

c - 2.4 x 10^7 spleen cells from normal nude mice/ml/culture.

d - 5.0 x 10^7 thymus cells from 38 day old Balb/c mice treated from day 0 to day 37 with normal rabbit serum.

e - 5.0 x 10^7 thymus cells from 38 day old Balb/c mice treated from day 0 to day 37 with rabbit anti-μ (mouse) serum.

f - 2.4 x 10^7 spleen cells from 38 day old Balb/c mice treated from day 0 to day 37 with normal rabbit serum.

g - 2.4 x 10^7 spleen cells from 38 day old Balb/c mice treated from day 0 to day 37 with rabbit anti-μ (mouse) serum.

cell for suppression of the immune response to SE is the B cell. With the high titers of circulating anti-μ found in suppressed animals, it seems logical to expect that if the thymus cell is affected in its ability to cooperate in the immune response by anti-μ, then is should be affected in these highly suppressed animals. The evidence thus indicates that a μ chain is not a part of the receptor site on thymus cells or that it is not accessible to anti-μ.

DISCUSSION

Studies involving spleen cell cultures treated with anti-theta serum (48) or prepared from mice which were thymus-deprived by neonatal thymectomy (2) or by adult thymectomy followed by irradiation and reconstitution with bone marrow (3,4), have shown a requirement for thymus-derived cells in the in vitro immune response to heterologous erythrocytes. With these observations and the observation by Reed and Jutila (17) that athymic mice did not respond to heterologous erythrocytes in vivo, the inability of nude spleen cell cultures to respond to SE in vitro was totally as expected. The low response of nude spleen cells in vitro to SE, as previously reported (42), was confirmed by others (49,50,51,52). The only information contradictory to this is a reference to "unpublished observations" in an article by Watson et al. (53).

The unresponsiveness of nude cell cultures was relatively uniform in contrast to the considerable variation in response observed in cultures obtained from the spleens of neonatally thymectomized mice (2). In the latter case, a significant proportion of cultures responded to SE as well as those from normal animals and about 60 per cent of the cultures gave lower responses than normal cultures. This difference between nude spleen cell cultures and cultures prepared from the spleens of neonatally thymectomized mice may be due to seeding of cells from the thymus prior to its removal at birth. Indirect evidence that cell

seeding may occur from the thymus prior to birth is found in a recent report (54) that phytohaemagglutinin stimulated thymocytes from an 18 day mouse embryo to a greater extent than at any other time in gestation or later life.

The response of cultures prepared from thymus-deprived animals was increased by various procedures including thymus grafting of the spleen donor (3), addition to the cultures of either thymus cells (4), or normal (2,4) or irradiated spleen cells (2), or by culturing in various cell supernatants (32). In this study, in vitro cultures prepared from nude spleens were stimulated to respond to SE by the addition of Balb/c or LM thymus or spleen cells, but irradiation was observed to inhibit cells from cooperating within the nude cultures, and supernatants were not observed to confer the ability to respond to SE. Following the report (42) that thymus and spleen cells from normal animals were capable of establishing an immune response to SE in nude spleen cell cultures, similar observations using thymus cells (49,50, 51) and spleen cells (52) were reported by others.

The inability of supernatants from thymus and normal spleen cell cultures to establish an immune response to SE was first thought to be due to a total absence of T cells in nude spleens. Cultures prepared from thymus-deprived mice may contain a small number of residual T cells that were seeded prior to birth and thymectomy, were irradiation

resistant, or were seeded to bone marrow and subsequently injected along with bone marrow cells into irradiated animals. The stimulation of these residual T cells could account for the success of supernatants in reconstitution attempts. Although this theory remains tenable, a recent report (55) indicates that an immune response to SE can be obtained in nude spleen cell cultures by combining the cell supernatants from two different strains of mice (CBA and C57Bl/6) and culturing nude spleen cells in this combination. It is difficult to understand the mechanism involved in this situation because supernatants from cells of either strain alone were not observed to give this effect.

The degree of compatibility required for cellular cooperation during immune responses in vivo and in vitro may not be the same. Kindred (56) has observed in in vivo studies involving backcrossing that lasting immune responsiveness to SE was obtained only from donor animals "related" to the nude recipients. In this dissertation, and as previously reported (42), Balb/c thymus was observed to be as effective in vitro as was LM thymus, and Bösing-Schneider and Kindred (51) have recently reported using thymus cells from various sources in establishing an in vitro immune response to SE in nude spleen cell cultures. Additional observations by Isaak (57) have also indicated that relatedness may not be essential in vivo either, for thymus glands from strains other than the one in which the nude is bred can confer the

ability to produce antibody to SE. In this dissertation (Table VIII), cultures prepared from nudes grafted with Balb/c or LM thymuses and used months later as spleen cell donors were observed to respond in vitro to SE indicating that lasting immune responsiveness has been obtained by using thymus glands from these sources. This question could be further investigated by grafting nudes on the Balb/c background with thymus glands or thymus cells from other strains and species, and later assaying their ability to respond to SE in vitro.

A response to SE was established in nude spleen cell cultures by the addition of CBA, Balb/c, and LM thymus and spleen cells and by the addition of C57Bl/Ks lymph node cells. To determine if heterologous lymphoid cells would establish an immune response in nude spleen cell cultures, rat thymus and spleen cells were added. Heterologous thymus and spleen cells did not establish an immune response to SE in nude spleen cell cultures, nor did rat spleen cells respond to SE in cultures.

Neuraminidase was used in a single attempt to alter the cell membrane surface of cells. The bone marrow is the original source of the cells contained in the thymus, and it seemed possible that the thymus could impart activity by 'baring' determinates previously hidden. Neuraminidase, which cleaves salicylic acid from membranes, was added to nude spleen cell cultures to ascertain if determinates were made

available that would enable cells to respond to SE. The results were negative under the conditions utilized, but it has recently been reported that neuraminidase significantly enhanced the blastogenic response of sensitized human lymphoid cells to various mitogens and antigens (58).

The results in this study strongly indicated that the adherent cell contained in nude spleens is functional. The combination of adherent cells from nude spleens and nonadherent cells from Balb/c spleens responded to SE, and low numbers of nonadherent cells from Balb/c spleens were capable of establishing an immune response to SE in nude spleen cell cultures. Additional evidence is found in the ability of nude spleen cell cultures to respond to the antigen LPS when the whole *E. coli* cell is added to cultures. This evidence is indirect in that an adherent cell has not been demonstrated as an absolute requirement for the response to this antigen.

Spleen cells from nude mice do not respond to SE in vitro, but by obtaining a response to LPS using whole heat killed *E. coli* cells (C-LPS), evidence is given that nude spleen cells can respond in vitro to an antigen that does not require the participation of thymus-derived cells. The in vivo responses to LPS and C-LPS have previously been shown to occur in the absence of thymus-derived cells (13,14,45). The results described in this study show that an in vitro response to C-LPS can be obtained, and additionally, that thymus-derived cells are not

required. The results and conclusions are similar to those of Sjöberg (59). In his study, spleen cell populations which had been depleted to T cells by the use of anti-theta serum and complement retained the ability to respond to LPS from _E. coli_ when the whole bacterial cells were used as antigen. Utilization of cell cultures prepared from the spleens of nude mice, as in the experiments described in this dissertation, and as previously reported (46), avoided the possibility of small numbers of T cells escaping destruction by anti-theta serum and complement.

Although an in vitro immune response to soluble LPS was not obtained in spleen cell cultures from normal or nude mice, it is possible that with proper manipulation a response could be obtained. Andersson et al. (60), using normal spleen cell cultures, have reported a response to soluble LPS. However, the response was low and was obtained over a narrow LPS concentration range. As the LPS concentration was increased, the number of PFC to LPS did not increase in parallel with the increased DNA synthesis (60).

That nude spleen cell cultures can respond to C-LPS is good evidence that this in vitro response does not require the participation of thymus-derived cells. This does not rule out the possibility of thymus-derived cells having a regulatory function, as in the response to pneumococcal polysaccharide (61,62), although current evidence

indicates that the in vivo immune response to LPS is not under thymic regulation (63,64). The evidence in this study also indirectly supports the idea that LPS is not under thymic control or regulation, for nude and normal spleen cell cultures responded in a similar manner. If thymus-derived cells possessed a regulatory function, one might expect an increased, or more likely, a decreased response to LPS in cultures containing T cells.

In this study, the addition to nude spleen cell cultures of SE coated with LPS did not establish an immune response to SE. That this was not successful may be due to the fact that only small amounts of LPS coat the SE (47). LPS in the soluble state has been observed (65,66) to induce an immune response to SE in nude spleen cell cultures. The concentration of LPS necessary to establish the response is fairly low, with 5 µg (65) and 10 µg (66) reported as being optimal. With this information, it seems necessary to question if the results observed by Watson and Epstein (53) in obtaining an in vitro response to SE in nude spleen cell cultures were possibly due to endotoxin, or some unknown B cell mitogen present in the cell and media (especially the calf serum) preparations. Microgram quantities of LPS stimulated an immune response to SE in nude spleen cell cultures (65,66), and the possibility exists that even smaller amounts of other compounds might exert the same effect.

The radiosensitivity of T cells is of considerable debate. Kettman and Dutton (67) reported that the carrier-effect of T cells was not only radioresistant, but that irradiation (1000 or 4000 R) enhanced the immune response, while Anderson, Sprent, and Miller (68) recently reported that carrier-primed T cells were radiosensitive. Using thymus-deprived mice and an in vitro primary response to SE, Hirst and Dutton (2) and Munro and Hunter (4) have reported that the cooperating T cell obtained from mouse spleen cells is resistant to 1000 R, while Roseman (69) observed that the in vitro immune response was suppressed in cultures prepared from animals which had received 250 R. Haskill, Pyrt, and Marbrook (70) using density gradient separation of cells observed that the cooperating T cells from spleen were radiosensitive to 500 R.

In this study, thymus cells and thymus-derived spleen cells were observed to be radiosensitive. This conclusion is supported by the report by Bösing-Schneider and Kindred (51) who observed that 1000 R treatment of thymus cells inhibited their ability to establish an immune response in nude spleen cell cultures. Contradictory to this, Watson et al. (65) reported that 2000 R had no effect on the ability of thymus cells to establish a response in nude spleen cell cultures. A possible explanation may exist for this reported difference in the ability of irradiated thymus cells to cooperate in nude spleen cell cultures. Watson and co-workers (65) did not report the procedure

used to irradiate the thymus cells nor the treatment of cells following irradiation. If the thymus cells were not washed following irradiation, a thymus cell factor or factors may have been present in the supernatant that was responsible for establishing an immune response. This explanation would not hold for the difference observed in carrier-primed systems (67), for the irradiated cells were reported washed as they were in this dissertation.

In vivo (30) and in vitro (71,72) immunosuppression studies with heterologous anti-immunoglobulin heavy chain antisera show that B cells are affected. The question as to whether the T cell is also suppressed in these animals and cultures has not been satisfactorily answered. Cell transfer studies using suppressed animals as donors and lethally irradiated recipients have not answered the question as to thymus cell suppression (30). The in vivo suppression of athymic mice (30) to LPS gave good evidence for the involvement of the B cell in anti-μ suppression; but since LPS does not require the participation of T cells, it did not answer the question of T cell involvement. In vitro studies have previously either involved the suppression of normal spleen cells with specific heavy chain antisera (71) or used thymus-derived animals and nonspecific antiserum (72). Pierce et al. (71) reported a thorough investigation of the kinetics of in vitro suppression, but did not investigate the target cell of suppression. Doria et al. (72) approached

the question of target cell suppression by using neonatally thymectomized animals as a source of B cells and thymus cells from normal animals. The antiserum used to treat cells was undiluted rabbit serum from animals immunized with whole mouse serum (72). This is in contrast to the highly specific high titered rabbit anti-μ serum used in this investigation which suppressed the in vitro immune response to SE to less than 10 per cent of normal at a 1:200 dilution and totally suppressed the response to SE at a 1:100 dilution. Other studies (71,72) seldom reported greater than a 90 per cent reduction in response in vitro.

The data obtained using in vitro treated thymus cells and thymus cells from animals suppressed from birth strongly indicated that the thymus cell is not suppressed in its ability to cooperate with B cells in vitro. B cells from Balb/c mice treated with anti-μ serum from birth or B cells from Balb/c or nude spleens treated in vitro with anti-μ serum were suppressed in vitro and unable to respond to SE. The conclusions using this more defined system and highly specific antiserum are similar to those of Doria et al. (72). Cell cooperation is prevented by treating the B cell population with anti-μ serum and thymus cells appear to lack demonstrable receptor molecules for anti-μ, or μ comprised receptor sites on thymus cells are not involved in cell cooperation in the immune response to SE.

The allogeneic effect is a phenomenon in which specific B lymphocytes become readily activated in the absence of antigen-specific helper (cooperating) T cells by virtue of a direct interaction with histoincompatible T lymphocytes recognizing surface antigen differences on such B cells. Two excellent review articles are to be found on this topic (73,74).

Because our athymic (nude) mouse is not an inbred strain and is on approximately the fourth generation backcross in the Balb/c line, the role of the allogeneic effect must be considered when an immune response is established to SE, or any other thymus dependent antigen, using LM or Balb/c thymus or spleen cells. In the majority of studies reported within, Balb/c animals were used as cell donors because that was the background on which the nude had been bred and because this avoided genetic variation, as would have been expected if LM mice were utilized. This does not in any manner avoid the possibility of the allogeneic effect occurring in this system and exerting a role in establishing the immune response to SE in nude spleen cell cultures. In experiments to investigate the idea of the allogeneic effect expressing itself in this system, C57Bl/Ks lymph node cells were used to establish an immune response in nude spleen cell cultures. The C57Bl/Ks mouse has the same $H\text{-}2^d$ locus as does Balb/c, but the two strains differ at other histocompatibility loci. If C57Bl/Ks lymph

node cells had been more effective at establishing a response than Balb/c lymph node cells, one would have had evidence for the allogeneic effect occurring. As enhancement was not observed, no conclusions were possible concerning the allogeneic effect in the system. The question will remain unanswered until an inbred nude animal is available.

The evidence from all aspects investigated indicated that nude mice have functional bone marrow-derived cells. Nude spleen cell cultures responded to an antigen that does not require T cells, and a response to an antigen requiring T cells can be obtained by the addition of thymus cells to cultures. The absence of T cells in nudes and the apparent normalcy of the B cell population provides a useful tool in the study of cell interactions.

ADDENDUM

Two very recent articles have appeared that are indirectly of importance to this dissertation. Talmage and Hemmingsen (75) have reported that there is a third nonadherent cell required for the in vitro immune response to SE. This third cell is bone marrow-derived and is distinguished from the precursor of the PFC by being more sensitive to actinomycin D, less sensitive to irradiation, being first to repopulate the spleen of irradiated mice injected with bone marrow, and not increased in numbers after immunization. Since thymus cells are capable of establishing an immune response to SE in nude spleen cell cultures, nude spleen must contain this second nonadherent cell. The second article by Komuro and Boyse (76) reports the in vitro differentiation of nude lymphocytes into T lymphocytes as defined as cell bearing the thymus associated antigens TL and Thy-1. Induction of these antigens from nude spleen and embryonic liver occurs with addition of a mouse thymus extract. No evidence is given in this report (76) as to whether these converted T cells have normal T cell immunological capacities.

SUMMARY

Spleen cell cultures from athymic (nude) mice were found to be unresponsive to sheep erythrocytes (SE) in vitro. The addition of 5.0×10^7 thymus cells from Balb/c or littermate animals to nude spleen cell cultures enabled these cultures to respond to SE. An in vitro response to SE could also be obtained by addition of normal spleen cells from Balb/c or littermate mice. As few as 2.4×10^5 normal spleen cells established an immune response in nude spleen cell cultures. Cell cultures prepared from nude mice grafted with thymus glands 2-5 months prior to being used as spleen donors responded to SE as well as Balb/c spleen cell cultures.

Using various combinations of adherent and nonadherent cells from nude and Balb/c spleens, the adherent cell population of nudes was seen to be functional in the immune response to SE. Heterologous thymus and spleen cells from rats failed to establish an immune response to SE in nude spleen cell cultures.

Thymus cells were observed to be radiation sensitive, with a dose of 400 rads effectively inhibiting their ability to establish an immune response to SE in nude spleen cell cultures. Thymus-derived spleen cells, obtained from mice lethally irradiated and injected with thymus cells, were also found to be radiation sensitive, but not as sensitive as thymus cells obtained directly from the thymus.

Nude spleen was observed to have functional bone marrow-derived cells because nude spleen cell cultures responded in vitro to lipopolysaccharide (LPS) when whole heat killed E. coli cells (C-LPS) were used as the antigen. LPS has previously been reported to be an antigen that does not require the participation of thymus-derived cells to give an immune response. The obtainment of an immune response to C-LPS in nude spleen cell cultures and the ability to establish an immune response to SE with thymus cells indicated that while a deficiency of thymus-derived cells exists in nude mice, they have functional bone marrow-derived cells.

Suppression of the immune response to SE was obtained by an in vitro treatment of spleen cells for four hours with highly specific, high titered heterologous anti-IgM heavy chain antiserum or by addition of this antiserum to cell cultures. In vitro treatment (four hours) of Balb/c spleen cells totally inhibited the immune response to SE, and treatment of nude spleen cells inhibited the ability of thymus cells to establish an immune response to SE. Treatment of thymus cells with the antiserum did not affect their ability to establish an immune response to SE in nude spleen cell cultures. Furthermore, in vivo suppression of Balb/c mice from birth totally suppressed the response to SE in cell cultures prepared from their spleens, but did not affect the ability of their thymus cells to establish an immune response to SE in nude spleen cell cultures.

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