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

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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 IgM (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 C57l 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 4.8×10^7 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]		Indirect PFC[c]	
	culture	10^6	culture	10^6
Balb/c	1320	244	222	41
Littermate[d]	4800	678	2100	194

a - 2.0×10^7 spleen cells/ml/culture plus 3×10^6 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]		+SE[a]; PFC per[b]		No. of	
	culture	10^6	culture	10^6	Expt.[c]	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, 3×10^6 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 - $2.0\text{-}2.4 \times 10^7$ 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] 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 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]		+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
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 co-operate 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] Culture	-SE[a]; PFC per[b] 10^6	+SE[a]; PFC per[b] Culture	+SE[a]; PFC per[b] 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 2.4×10^6 N-4 cells to 2.4×10^7 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]		+SE[a]; PFC per[b]	
	Culture	10^6	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 (2.4×10^5)	7	1	357	42
Balb/c[c]	21	3	1320	220
Balb/c[c] + C57Bl/Ks lymph node cells (2.4×10^5)	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 (2.4×10^6)	46	8	5320	752
Balb/c[c]	21	3	1734	213
Balb/c[c] + C57Bl/Ks lymph node cells (2.4×10^6)	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 cooperating 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-2^k; Balb/c, H-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 - $2.0\text{-}2.4 \times 10^7$ cells/ml/culture.

d - 5.0×10^7 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.