Reconstitution of the *In Vitro* Immune Response of Congenitally Thymusless (Nude) Mice[1] (36500)

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The *in vitro* immune response to heterologous erythrocytes has become a useful system in studies of cell interactions in the immune response. By controlling *in vitro* the numbers and types of cells that are allowed to interact, at least three cell types have been shown to be required to generate a response to heterologous erythrocytes: an adherent cell, a thymus-derived nonadherent cell (T-cell), and a bone marrow-derived nonadherent cell (B-cell) (1).

Cultures prepared from the spleens of thymus-deprived 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 bone marrow (5, 6).

We believe these objections are largely alleviated by using cell cultures prepared from the congenitally thymusless (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 in 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 thymusless mouse would thus seem to be a useful tool in *in vitro* studies of cell interaction.

We describe in this report experiments which show that "nude" spleen cell cultures fail to respond to sheep erythrocytes (SE) unless the cultures are reconstituted[2] with normal thymus cells or normal spleen cells.

Materials and Methods. Mice. Congenitally thymusless mice (nu/nu), hereafter described as nude, and phenotypically normal littermate mice (+/nu and +/+) were the offspring of heterozygous animals obtained by crossing Re+/+ nu males (13) with females from our specific pathogen-free (SPF) Balb/c colony. Balb/c mice were used as a source of thymus and spleen cells and as controls. Littermate mice 7 to 14 weeks of age and of the same sex were used in individual experiments. All mice were given sterilized Purina 5010C feed and acidified–chlorinated water (14). Nude mice were continuously housed with normal littermates to delay the onset of wasting and improve the general health of the nude animals.

Cell suspensions. Cell cultures (1 ml) were

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[2] The authors point out that the term "reconstitution" is not entirely appropriate because this implies the previous ability to respond. This is not the case in the nude animal, which has not previously possessed the capacity to give an immune response to heterologous erythrocytes. Because the term "reconstitution" has previously been used in systems utilizing thymus-deprived animals, the term is retained in this report to refer to the establishment of an immune response to SE in cell cultures prepared from the spleens of nude mice.

prepared from the spleens and thymuses of mice according to the technique of Mishell and Dutton (15). Spleen cell cultures contained 2.0–2.4 $\times$ 10^7 cells/ml and thymus cultures contained 5.0 $\times$ 10^7 cells/ml. Sheep erythrocytes were added to the cultures from sheep 1786 (Colorado Serum Co.) and the plaque-forming cell (PFC) response on the fifth day of incubation was determined by a slide modification of the Jerne plaque assay (15). Slides were incubated 2 hr at 37° before the addition of complement (1:10) and 2 hr after complement was added.

In experiments to establish an immune response in nude spleen cell cultures the number of nude spleen cells cultured was held constant (2.0–2.4 $\times$ 10^7). To each such culture were added 2.4 $\times$ 10^4 to 2.4 $\times$ 10^7 littermate or Balb/c spleen cells, or 5.0 $\times$ 10^7 littermate or Balb/c thymus cells. In preparing thymus cell suspensions, special care was taken to exclude the parathymic lymph nodes (16).

Results. The PFC response to SE by nude spleen cell cultures was low when compared to either littermate or Balb/c responses (Table I). The background to SE was also found to be quite low in nude spleen cell cultures. This confirms our previous results *in vivo* (17), showing that the nude animal has an impaired immune response to SE. Preliminary experiments established the cell levels used as optimal.

Thymus cells from littermates or Balb/c mice were added to nude spleen cell cultures to determine if reconstitution of nude spleen cell cultures was possible. Balb/c thymus cells were found to markedly enhance the PFC response of nude spleen cell cultures (Table I). Thymus cells alone were found to be unresponsive *in vitro* and the addition of syngeneic thymus cells to Balb/c spleen cell cultures had little effect on the number of PFC per culture (due to the large number of thymus cells added the number of recovered cells was high, resulting in low PFC per 10^6, thus making the PFC per culture response of greater significance) (Table I). Results with littermate thymus cells were similar to those observed with Balb/c thymus cells.

Balb/c 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 reconstituted nude spleen cell cultures. The results in Table II show that Balb/c spleen cells in low concentration do not respond *in vitro*. The

TABLE I. *In Vitro* Response of "Nude," Littermate and Balb/c Spleen Cell Cultures and Reconstitution of Nude Spleen Cell Cultures with Balb/c 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
Littermate spleen[e]	56	4	1955	284	5	13
Balb/c spleen[e]	104	30	1968	320	4	19
Balb/c thymus[f]	0	0	0	0	3	9
Nude spleen[d] + Balb/c thymus[f]	130	20	2533	130	5	13
Balb/c spleen[d] + Balb/c thymus[f]	282	20	2595	197	4	19

[a] — SE, sheep erythrocytes not added to cultures; + SE, sheep erythrocytes added to cultures.
[b] Mean number of direct plaque-forming cells per culture or per 10^6 nucleated cells recovered.
[c] Number of experiments.
[d] Total number of mice used in experiments.
[e] 2.0–2.4 $\times$ 10^7 cells/ml/culture.
[f] 5.0 $\times$ 10^7 thymus cells/ml/culture.

TABLE II. *In Vitro* Reconstitution of "Nude" Spleen Cell Cultures with Balb/c Spleen 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[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 I.

addition of as few as 2.4×10^5 normal Balb/c spleen cells however, resulted in reconstitution of nude spleen cell cultures. The resulting PFC response of these reconstituted nude spleen cell cultures was greater than Balb/c spleen cells alone. The addition of as few as 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 cells alone, and the highest PFC production occurred upon the addition of $2.4–4.8 \times 10^6$ Balb/c spleen cells to nude spleen cell cultures. Similar results were also observed with littermate spleen cells, but because of genetic variation among the littermates the inbred Balb/c mice were used for most reconstitution studies.

Discussion. Studies involving spleen cell cultures treated with anti-theta serum (18) 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. An impaired immune response was observed in these T-cell deficient cultures. The response of such cultures, however, was increased by various procedures including thymus grafting of the spleen donor (3) or addition to the cultures of either thymus cells (4), thymus-derived spleen cells (18), or normal (2, 4) or irradiated spleen cells (2).

In this study, *in vitro* cultures prepared from spleens of nude mice were found uniformly unresponsive to SE, whereas littermate and Balb/c spleen cell cultures responded well (Table I). This supports the *in vivo* work describing impaired production of hemagglutinins, hemolysins, PFC and rosette-forming cells by nude mice (17).

Because nude mice are congenitally thymusless, thymus cells from Balb/c or littermate mice were added to cultures of nude spleen cells in attempts to replace the deficient cell type. Large numbers of thymus cells markedly increased the PFC response of nude spleen cell cultures, whereas the addition of syngeneic thymus cells to Balb/c spleen cell cultures had little effect on the PFC response (Table I).

The consistent reconstitution of *in vitro* nude spleen cell cultures with Balb/c thymus cells is of interest. Kindred (19) has recently observed, in *in vivo* studies involving

backcrossing, that lasting immune responsiveness to SE was obtained only if thymus cells came from donor animals "related" to the nude recipients. Perhaps the degree of compatibility required for cellular cooperation during immune responses is not the same in *in vitro* and *in vivo* systems.

Since spleen as well as thymus contains thymus-derived cells (20), spleen cells from normal Balb/c mice were used in attempts to reconstitute nude spleen cell cultures (Table II). As few as 2.4×10^5 Balb/c spleen cells were effective in reconstituting cultures of nude spleen cells. Littermate spleen cells also reconstituted nude spleen cell cultures. The addition of Balb/c spleen cells to cultures of nude spleen cells resulted in a larger PFC response than the response of cultures of normal Balb/c spleen cells alone (Table II). This was observed even when low numbers of Balb/c spleen cells were added to nude spleen cell cultures. A possibly related observation has been reported by Hirst and Dutton (2), who found that spleen cell cultures from neonatally thymectomized mice are more effectively reconstituted by irradiated allogeneic cells than by irradiated syngeneic cells.

The unresponsiveness of nude spleen cell cultures and the reconstitution of these cultures with thymus cells indicates that while a deficiency of thymus-derived cells exists in nude mice they have functional bone marrow-derived cells. The unresponsiveness of nude cell cultures was uniform, in contrast to the considerable variation in response observed in cultures prepared 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% of the cultures gave lower responses than normal cultures. This difference between nude 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.

These studies show that the congenitally thymusless mouse is a useful system for *in vitro* studies of cell interactions in the immune response. It will be of special interest to study the *in vitro* response of nudes to alleged "thymus-independent" antigens.

Summary. Spleen cell cultures from congenitally thymusless (nude) mice were found to be unresponsive to 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 mice. As few as 2.4×10^5 Balb/c spleen cells established an immune response in nude spleen cell cultures. The reconstitution of nude spleen cell cultures with thymus cells indicates that while a deficiency of thymus-derived cells exists in nude mice, they have functional bone marrow-derived cells.

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