Immune Response of Congenitally Thymusless Mice to Heterologous Erythrocytes[1] (36337)

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Experiments involving neonatal thymectomy have demonstrated the central role of the thymus in the development of immunological competence (1). Since these experiments do not define the contribution of the thymus prior to its removal at birth, there has been great interest in the autosomal recessive mutant, *nude* (*nu*), described by Flanagan (2). "Nude" mice are hairless, have low fertility, poor viability and, as shown by Pantelouris (3), fail to develop thymuses. In nude mice, the blood lymphocyte count is low (4) and the thymus-dependent areas of the lymph nodes, spleen, and Peyer's patches are deficient in lymphocytes (5) and in cells bearing the θ antigen (6). Allografts and xenografts are accepted indefinitely by nudes (4, 7–10).

The present report describes the response of congenitally thymusless mice (nudes) to heterologous erythrocytes, as measured by the production of hemolysins, hemagglutinins, plaque-forming cells and rosette-forming cells.

Materials and Methods. Mice. The homozygous (*nu/nu*) mice, which are congenitally thymusless and are hereafter described as nude, and littermate control (+/*nu* and +/+) mice were the offspring of heterozygous animals obtained by crossing *Re* +/+ *nu* males (8) with females from our specific pathogen-free (SPF) Balb/c colony. All mice were given sterilized Purina 5010C and acidified-chlorinated water (11). In nude mice reared under SPF conditions, the onset of wasting is delayed and we have been able to obtain offspring by mating nude males and females, and by mating nude males and females with Balb/c mice. Nude mothers usually do not suckle their young more than a few days. The general health of nude mice is also improved if nudes are continuously housed in cages with normal mice. The mice used in these experiments were 7 to 14 weeks of age.

Immunizations. Mice were given a single intraperitoneal injection of sheep erythrocytes (SE), washed 3 times in phosphate-buffered saline, in doses of 10^7, 10^8, 10^9, or 10^{10} cells.

Immunological assays. All immunological assays were done on individual mice 5 days after immunization. Mice were bled from the retro-orbital sinus; and twofold serial dilutions of serum samples were prepared in modified barbital buffer (12). Agglutinin (HA) assays were done as described by Adler (13). After centrifuging the tubes and recording the agglutinin titer, the SE were resuspended and 0.1 ml of a 1:10 dilution of guinea pig complement was added to each tube. Hemolysin (HL) titers were determined after incubation at 37° for 1 hr.

The plaque-forming cell response was determined by the slide test described by Mishell and Dutton (14). Slides were incubated 2 hr at 37° before the addition of complement and 2 hr at 37° after complement was added. In some experiments indirect plaques were developed using a goat antimouse γG serum (15).

The rosette technique was done essentially as described by Biozzi *et al.* (16). The entire procedure was done at 4°. To perform the test, 0.1 ml of 5% SE or 2% chicken erythrocytes (CE) and 0.1 ml of a mouse spleen cell preparation, containing 3–6 $\times$ 10^6 nucleated

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TABLE I. Immune Responses of Congenitally Thymusless ("nude") Mice and Their Phenotypically Normal Littermates.

Mice	Dose of SE	Mean titer[a] HA		Mean titer[a] HL		Direct[b] PFC/10^6	RFC[c] × 10^3/10^6 formed with SE		RFC[c] × 10^3/10^6 formed with CE	
Nude	None	3	(4)[d]	0	(4)[d]	0.7	(10)[d]	0.29	(8)[d]	5.70
Normal		0	(5)	0	(5)	0.5	(9)	0.64	(7)	6.91
Nude	10^7	4	(7)	0	(6)	5	(7)	0.31		
Normal		106	(6)	400	(6)	67	(6)	2.68		
Nude	10^8	16	(13)	163	(13)	12	(12)	0.41		5.74[e]
Normal		926	(14)	2903	(14)	225	(13)	15.91		6.30[e]
Nude	10^9	158	(8)	700	(8)	70	(7)	0.51		
Normal		1960	(8)	5440	(8)	127	(7)	23.69		
Nude	10^{10}	194	(5)	448	(5)	69	(5)	0.42		
Normal		800	(4)	1920	(5)	225	(4)	8.90		

[a] Mean hemagglutinin (HA) or hemolysin (HL) titer.

[b] Mean number of direct plaque-forming cells per million nucleated spleen cells.

[c] Mean number of rosette-forming cells times 10^3 per million nucleated spleen cells when tested with sheep erythrocytes (SE) or chicken erythrocytes (CE).

[d] Number of mice studied indicated in parentheses.

[e] Specificity controls (1 animal/group).

cells, were added to 0.8 ml of buffered saline (16) in a 100 × 12 mm tube closed with a rubber stopper. The mouse spleen cells, SE, and CE were washed thrice in buffered saline prior to use in the test. Tubes were left standing at 4° overnight, and then the cells were resuspended by gently rotating the tubes vertically at 100 rpm for 8 min on an Eberbach rotator. Rosettes were counted in a hemacytometer. A rosette consisted of a nucleated spleen cell with 5 or more CE or 8 or more SE adhering to its surface. The number of nucleated cells in each spleen cell preparation was determined after lysis of SE with an aqueous solution of 1% acetic acid. Results were expressed as rosette-forming cells (RFC) × 10^3/10^6 total nucleated spleen cells.

Statistics. The p values were calculated according to Student's t test. In comparison of the means of any two groups of observations, a significance level of .05 was chosen.

Results. HA and HL activity was detectable in nude mice at SE doses of 10^8 cells and above (Table I). However, the HA and HL responses of nude mice were significantly lower than the responses of their phenotypically normal littermates at all antigen doses studied.

The PFC response of nudes was also lower than the response of normal littermates (Table I). This difference was most pronounced at SE doses of 10^7 and 10^8; the difference between nudes and controls was not significant at doses of 10^9 and 10^{10}. However, in experiments involving 6 nudes and 6 littermate controls, indirect plaques were developed using a goat antimouse γG serum. This resulted in increased numbers of PFC in controls but not in nudes, and the difference between nudes and controls was then significant at the higher doses of SE.

The number of RFC to SE (Table I) observed in nudes compared to their littermate controls differed significantly ($p < .001$) in all immunized groups except the group which received 10^7 SE ($.1 > p > .05$). The number of RFC in all immunized control groups was significantly ($p < .001$) higher than the background control level, except in the control group immunized with 10^7 SE ($.1 > p > .05$). In contrast, the number of RFC in immunized nudes did not differ significantly ($p > .05$) from the background level observed in unimmunized nudes in any case. RFC formation was inhibited by pretreatment of nude or control mouse spleen cells with rabbit antimouse γ-globulin.

Background RFC to SE and CE were observed in all nudes examined. It was possible that these RFC were of maternal origin, since the nudes were the progeny of heterozygous (*nu*/+) females which have normal thymuses. Thus, homozygous nude females were mated with homozygous nude males and the progeny, all nude, were foster-nursed on heterozygous females. Two of these progeny nudes were examined for background RFC at 6 weeks of age. The mean number of background RFC to SE ($0.31 \times 10^3/10^6$) and CE ($7.01 \times 10^3/10^6$) in these nude mice was similar to that observed in nude mice born to females with normal thymuses (Table I).

Discussion. The HA and HL production in response to SE injection was significantly less in nude mice than in their littermate controls at all antigen doses studied (Table I). Impairment of antibody production by nudes has been reported by Kindred (10) who observed that nudes produced a small amount of hemolyzing antibody in response to SE, but, in contrast to our observations, failed to produce hemagglutinating antibody. Pantelouris (7) reported that hemolysin activity in response to SE injection was detectable in nudes but was much lower than in normal animals.

The immune impairment of nudes could be partially overcome by increasing the antigen dose. However, antibody production under such conditions was a less efficient process than antibody production in the presence of thymus-dependent cells, since nude mice injected with 10^9 or 10^{10} SE did not respond as well as normal animals given 10^8 SE (Table I).

The direct PFC response of nude mice was also lower than the response of normal littermates. This difference was most apparent at lower antigen doses (Table I). The use of a developing antiserum at the higher antigen doses resulted in an increased total PFC response in normal mice but not in nudes, an observation which supports the contention that γG antibody production to SE is more dependent on the presence of thymuses than is γM antibody production (17).

It is of singular interest that background RFC were observed in unimmunized nude mice. On the other hand, the number of RFC in the spleens of nudes was not increased significantly by SE injection at any dose studied, whereas normal littermates responded strongly (Table I). This suggests that "immune" RFC and thymus-dependent or that thymus-dependent cells are required for the development of immune RFC. It is relevant that others (18) have found that many RFC in immunized mice are inhibited by anti-θ serum.

Since nude mice do not respond to SE by increased RFC production, the presence of background RFC in unimmunized nudes is of special interest. A maternal origin of the RFC in unimmunized nudes is unlikely since RFC were observed in young nudes born to nude females. Additionally, it is unlikely that the RFC observed in unimmunized nudes represent RFC formed in response to inapparent stimulation with cross-reacting antigens (19), since nudes failed to form increased numbers of RFC in response to injection with a wide range of SE concentrations. It is possible, however, that the RFC in unimmunized nudes represent a response to inapparent stimulation by cross-reacting antigens which are thymus-independent antigens.[2] We suggest that the background RFC in nude mice represent clones of cells of bone marrow origin which have surface receptors for SE and develop in the absence of antigenic stimulation. Additional studies of RFC in nude mice should increase our knowledge of the nature of RFC.

Summary. Hemagglutinin and hemolysin production by congenitally thymusless ("nude") mice in response to intraperitoneal injection of 10^7, 10^8, 10^9, or 10^{10} sheep erythrocytes (SE) was significantly lower than in normal littermate controls. The plaque-forming cell response of nudes was also impaired. Rosette-forming cells (RFC) were observed in unimmunized nude mice. Injection of SE did not result in the formation of increased numbers of RFC in nude

[2] We have recently shown that nudes make strong immune responses to *E. coli* lipopolysaccharide and pneumococcal polysaccharide.

mice, whereas littermate controls responded strongly.

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