INDUCTION AND ABROGATION OF UNRESPONSIVENESS IN NUDE MOUSE CELLS*

By RITA BÖSING-SCHNEIDER and MARGARETE HAUG

(From the University of Konstanz, Department of Biology, 775 Konstanz, Germany, Postfach 7733)

Nude mice, congenitally lacking a thymus and immunologically deficient in their response to T-dependent antigens, can be restored in vivo and in vitro by the addition of syngeneic or allogeneic T cells (1, 2). Restored animals respond to sheep erythrocytes (SRBC) with good antibody titers, whereas control mice, without T cells, cannot. However, when nude mice are pretreated with antigen (SRBC) and then several days later restored with thymus-derived cells, they are unable to respond to a second administration of antigen (3, 4).

It has been suggested that B cells are triggered to antibody production only if they receive two signals, whereas antigen stimulation alone without the relevant second signal would paralyze the B cell (5). On the other hand, induction of memory cells in nude or thymectomized animals has been demonstrated in several experiments (6–8).

In the present paper we studied the capability of overtly unresponsive nu/nu cells, to give an in vitro response in the presence of a T-cell-replacing factor (TRF)[1] derived from congeneic thymus cells. With this experimental design uncontrolled effects of additional T cells are avoided.

Materials and Methods

Animals. BALB/c-nu/nu mice were purchased through Bomholtgard, Ry, Denmark or derived from our own breeding stock (10th–12th backcross generation). BALB/c-Ig^b mice, 4- to 5-wk old, were used to produce activated T cells; mice 8–12-wk old were taken as a source of spleen cells for the production of a TRF.

Immunization and Cell Transfer. BALB/c nu/nu mice were immunized with a single dose of 3–5 × 10^8 SRBC at the age of 6–8 wk. Congeneic thymus-derived cells were given to these mice at different times after the injection of antigen. At varying times after the first or second injection, spleen cells were harvested, washed in Eagle's medium, and transferred to in vitro cultures for incubation in the presence or absence of a TRF.

Activated T Cells. Antigen-activated T cells were derived from the spleen of BALB/c-Ig^b mice which had been irradiated and injected with 5 × 10^7 syngeneic thymocytes and 3 × 10^8 SRBC 7 days before use.

Cell Culture Conditions. Spleen cell cultures from nu/nu mice were prepared according to the method of Mishell and Dutton (9) and incubated in the presence of 2-mercaptoethanol (10). SRBC were used as antigen at a dose of 5 × 10^6 SRBC per 10^7 cells. After 4 days of in vitro incubation in the presence or absence of a TRF, four culture dishes of one group were pooled and the number of antibody-producing cells was determined in triplicate with a modified Jerne plaque technique.

TRF. TRF was produced in BALB/c-Ig^b spleen cell cultures after stimulation by concanavalin

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[1] *Abbreviation used in this paper:* TRF, T-cell-replacing factor.

A (2 μg/ml) and applied to nude cell cultures according to the method of Schimpl and Wecker (11).

Hemagglutination. Mice were bled from the tail. Twofold serial dilutions of single sera in phosphate-buffered saline (pH 7.2) were made and an equal volume of a 1.5% suspension of SRBC was added to each dilution. The samples were incubated at room temperature for 2 h. The hemagglutination titer was read as the highest dilution giving positive hemagglutination.

Hemolysis. Direct hemolysis titers were determined with 5- or 10-fold serum dilutions to which an equal volume of SRBC (10%) was added. The samples were incubated at 37°C in the presence of guinea pig serum (1:6) for 1.5 h.

Irradiation. Irradiation of mice was performed with a Siemens Stabiliplan unit (Siemens, Stuttgart, Germany) at 250 kV, 15 mA with 0.5-mm Cu and 1-mm Al filters. Mice received a dose of 600 R.

Results

Triggering of Spleen Cells from Pretreated BALB/c Nude Mice by a TRF. Nude mice were injected with a dose of 3–5 × 10^8 SRBC at different times before transfer of their spleen cells to in vitro cultures where they were incubated for 4 days and tested for responsiveness against SRBC. In Table I it is shown that untreated cells can be stimulated in vitro by antigen only in the presence of TRF. After 4 days of incubation there is a several-fold increase in the number of antibody-producing cells compared to control cultures without TRF.

TABLE I
Triggering of Spleen Cells from Pretreated BALB/c Nude Mice by a TRF

	In vivo		In vitro		
Exp. no.	Injection (day 0)	Time of cell transfer to in vitro cultures	SRBC, 3 × 10^6 /culture (day 0)	TRF (day 0)	PFC*/10^6 cells (day 4 response)
		day			
I	–	–	–	–	23
	–	–	+	–	40
	–	–	+	+	210
	SRBC	7	+	–	25
	SRBC	7	+	+	180
II	–	–	–	–	20
	–	–	+	–	8
	–	–	+	+	313
	SRBC	11	+	–	2
	SRBC	11	+	+	220
III	–	–	–	–	7
	–	–	+	–	3
	–	–	+	+	250
	SRBC	18	+	+	205
IV	SRBC	3	+	–	5
	SRBC	3	+	+	593
	SRBC	6	+	–	5
	SRBC	6	+	+	228
	SRBC	9	+	–	3
	SRBC	9	+	+	242

* Mean values of triplicate determinations.

Spleen cells taken from nude mice pretreated with antigen, which are unresponsive in vivo, do not differ in vitro from untreated cells. They, too, can be activated in the presence of TRF to specific antibody production. The time of pretreatment seems to be unimportant in these experiments. They all give a response similar to control cultures.

Induction of Unresponsiveness. Since pretreatment of nude mice with antigen did not result in unresponsiveness of their cells in vitro, the possibility was tested that unresponsiveness in vivo might be due to the second injection of T cells given several days after pretreatment with antigen. Therefore the following experimental design was employed: mice first received a dose of antigen, and several days later received 10^7 activated T cells together with a second dose of antigen. At varying times after the last injection such mice were assayed in two ways: (*a*) their serum antibody titer was determined by hemagglutination in order to prove unresponsiveness in vivo (Table II); and (*b*) their spleen cells were transferred to in vitro cultures together with antigen and TRF in order to study the capability to respond in vitro (Table III).

The in vivo results in Table II are as follows: mice treated only with antigen do not differ significantly in their serum titer from untreated control cultures. They fail to respond to SRBC, but when these mice received syngeneic thymus lymphocytes at the same time as antigen the serum titer against SRBC rose several-fold. On the other hand, when activated T cells were given to pretreated mice several days later to induce a secondary response, the antibody titer resembled untreated control mice, that is mice were unresponsive to the second

TABLE II

Failure of Congeneic T Cells to Reconstitute BALB/c Nude Mice Previously Injected with SRBC

First injection	Second injection	Time of second injection	Titer of anti SRBC-antibodies at 8 days after the second injection, hemolysis/hemagglutination*
		day	
–	–	–	10/<11
–	–	–	<10/<11
SRBC	–	–	<10/11
SRBC	–	–	<10/22
SRBC	T‡ cells	0	50/175
SRBC	T‡ cells	0	100/352
SRBC	T§ cells	0	100/704
SRBC	T§ cells	0	50/88
SRBC	T§ cells + SRBC	6	<10/<11
SRBC	T§ cells + SRBC	6	<10/<11
SRBC	T§ cells + SRBC	7	10/11
SRBC	T§ cells + SRBC	7	10/22

* Geometric mean.

‡ Mice received 10^7 hydrocortisone-resistant thymus lymphocytes.

§ Mice received 10^7 antigen-activated T cells from irradiated donors which received 3–5 × 10^7 syngeneic thymus cells and 3 × 10^8 SRBC 7 days before harvest.

TABLE III

Failure of Triggering Pretreated Spleen Cells from BALB/c Nude Mice in the Presence of a TRF

Exp. no.	In vivo				In vitro		
	First injection (day 0)	Second injection	Time of second injection	Time of transfer to in vitro cultures	SRBC, 3×10^6/culture (day 0)	TRF (day 0)	PFC*/10^6 cells (day 4 response)
			day	*day*			
I	–	–			+	–	8
	–	–			+	+	847
	SRBC	Tact + SRBC	12	22	+	–	1
	SRBC	Tact + SRBC	12	22	+	+	63
II	–	–	–	–	+	–	2
	–	–	–	–	+	+	200
	SRBC	Tact + SRBC	12	22	+	–	44
	SRBC	Tact + SRBC	12	22	+	+	30
III	–	–	–	–	+	–	11
	–	–	–	–	+	+	485
	Thy (10^7)	–	–	12	+	–	22
	Thy (10^7)	–	–	12	+	+	8
	Thy (10^6)	–	–	12	+	–	5
	Thy (10^6)	–	–	12	+	+	63
IV	–	–	–	–	+	+	719
	Thy (10^7)	–	–	5	+	–	30
	Thy (10^7)	–	–	5	+	+	38

* Mean values of triplicate determinations.

administration of antigen. The data of in vitro cultures in Table III may be summarized as follows: (*a*) Cells from nude mice not pretreated respond well in vitro to the addition of antigen and TRF. (*b*) Prior injection of antigen, which leads to in vivo unresponsiveness, nonetheless does not prevent cells from being stimulated in vitro by antigen and TRF. (*c*) The regimen of prior injections of antigen followed by activated T cells together with antigen several days later not only leads to unresponsiveness in vivo, but also caused the cells to remain unresponsive in vitro; the unresponsive state was essentially not overcome by the addition of TRF.

Effect of Pretreatment with Thymus Lymphocytes. Since we found that priming of nude mice with antigen was not sufficient for the induction of in vitro paralysis to SRBC (Table I), but that the additional administration of activated T cells and antigen was essential, we investigated whether priming with antigen and activating the thymocytes by antigen were necessary conditions for unresponsiveness or whether thymus lymphocytes alone could bring about this effect. Therefore congeneic BALB/c-Ig^b thymocytes were injected at different times into nude mice before cells from these mice were transferred to in vitro cultures and stimulated with SRBC and TRF. As can be seen in Table III, pretreatment of BALB/c nude mice with 10^7 congeneic BALB/c thymocytes is followed by a defect in responsiveness to SRBC in vitro. Similar results were obtained when nude mice were injected with 10^6 thymocytes 12 days before explantation.

Failure of a Second Administration of Antigen to Induce Unresponsiveness In Vitro. In order to test if a higher dose or a second administration of antigen

would lead to unresponsiveness in vitro, nude mice received a 10-fold higher dose, namely 3–5 × 10^9 SRBC or in parallel experiments mice were treated twice with a dose of 3–5 × 10^8 SRBC without adding thymus lymphocytes. Several days later cells from these mice were transferred to in vitro culture. The results of these experiments are shown in Table IV. Either pretreatment of nude mice with a dose of 3–5 × 10^9 SRBC cells in vitro or two administrations of 3–5 × 10^8 SRBC at different times before transfer failed to induce unresponsiveness in the presence of TRF.

Discussion

Injection of nude mice with a T-dependent antigen results in nonresponsiveness when these mice receive syngeneic T cells and antigen 1 wk later (3, 4). It has been postulated that this status of immunological paralysis is not due to abolishment of the relevant precursor cells but to a transient inactivation of these cells; this was shown to be the case because paralysis could be overcome by treatment with supernates from paralyzed cells (4). Our data have shown that antigen-primed nude spleen cells can respond in vitro in the presence of TRF. If, however, T cells have been administered several days previous to in vitro culture, we observe paralysis also in vitro. Our in vitro experiments were performed without additional thymus-derived cells, thus avoiding possible suppressive or regulatory effects of such cells during in vitro culture. In this respect, the in vitro system allows a sharp separation between the condition of nude cells before and after administration of T cells, which is not possible in experiments conducted entirely in vivo. The data clearly show that treatment by antigen alone does not cause unresponsiveness. It is also obvious that the addition of activated T cells together with antigen or administration of thymocytes leads to an effective decrease in the capability to respond in vitro in the presence of TRF. One should stress that spleen cells from nude mice which had previously received activated T cells in vivo cannot respond in vitro to antigen with or without TRF, despite the fact that enough helper cells should have reached the spleen of these mice.

TABLE IV

Failure of a Second Administration of Antigen to Induce Unresponsiveness In Vitro

	In vivo			In vitro		
Exp. no.	First injection (day 0)	Second injection (day 7)	Time of transfer to in vitro cultures	SRBC (day 0)	TRF (day 0)	PFC*/10^6 (day 4)
			day			
I	3 × 10^8 SRBC	3 × 10^8 SRBC	11	+	–	67
II	3 × 10^8 SRBC	3 × 10^8 SRBC	11	+	+	1,385
III	3 × 10^9 SRBC	–	11	+	–	109
IV	3 × 10^9 SRBC	–	11	+	+	1,379
V	–	–	–	+	–	60
VI	–	–	–	+	+	803

* Means values of triplicate determinations.

Our results have a certain analogy to the reports of Gershon and Kondo (12), who found that pretreated spleen cells from thymectomized, lethally irradiated and bone marrow-grafted mice are unresponsive after the adoptive transfer only when given thymocytes and pretreated with antigen as well.

Since the capability of mounting an immune response is restored in nude mice when thymus-derived lymphocytes are injected simultaneously with the antigen, one can assume that the condition of the nude mouse is such that thymus-derived lymphocytes can enter different pathways of maturation, dependent on the presence or absence of the antigen.

Another possibility cannot be excluded, namely, that unresponsiveness in vivo, induced by antigen, is not related to unresponsiveness in vitro, induced by T cells. It is conceivable that antigen pretreatment activates a weak T-independent antibody production which may block further T-cell interaction in vivo. After transfer to cultures this blocking effect may be lost. A suppressive activity of T-cell-independent IgM on antibody production has been shown in nonresponder mice (13).

Summary

Nude mice were injected with antigen and T cells at different times to induce unresponsiveness to SRBC. Spleen cells derived from these mice were tested in vitro for the capability to produce antibody-forming cells against sheep erythrocytes in the presence of a T-cell-replacing factor. It was found that priming with antigen alone did not result in paralysis but a later injection of thymus-derived lymphocytes together with antigen results in unresponsiveness of these cells in vitro, provided there was an interval of several days between the in vivo administration of thymus lymphocytes and the explantations of cells to in vitro cultures.

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