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Infectious Immunological Tolerance

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Summary. Previous studies have shown that thymectomized lethally irradiated bone marrow grafted mice, reconstituted with thymocytes and pretreated with a large dose of sheep red blood cells (SRBC), are unable to respond to a subsequent immunizing injection of SRBC even after an inoculation of normal thymocytes. If, however, the mice are not thymocyte reconstituted prior to the pretreatment with SRBC, they can respond almost normally to an immunizing injection of SRBC if inoculated with normal thymocytes after the termination of antigen pretreatment.

In the present study the immunosuppressive effect of the presence of thymocytes during the antigen pretreatment was studied by adoptively transferring the spleen cells of the antigen pretreated mice to thymus-deprived chimeras. These spleen cells not only did not co-operate with normal thymocytes in the secondary hosts, but they also prevented the co-operation of normal thymocytes with normal bone marrow derived cells. Untreated spleen cells or treated spleen cells from mice not reconstituted with thymocytes did not affect cell co-operation in the secondary hosts. The abrogation of the co-operation in the secondary host was specific in that the addition of spleen cells did not affect the anti-horse red blood cell response. If the primary host made antibody as a result of the pretreatment, the transfer of their spleen cells did not prevent antibody production in the secondary host.

INTRODUCTION

We have recently reported that co-operation between lymphoid cells may play an important role in the induction of immunological tolerance (Gershon and Kondo, 1970). In those studies tolerance induction was studied in two groups of mice. Both groups were thymectomized, lethally irradiated and bone marrow grafted; one group was also given $1{\cdot}5 \times 10^7$ thymocytes along with the bone marrow. Both groups of mice were then given a large number ($2{\cdot}5 \times 10^{10}$) of sheep red blood cells (SRBC) over a 30-day period. Four days after termination of this tolerance induction schedule, neither group of mice could respond to an immunizing dose of antigen. However, if normal thymocytes were innoculated at the time of immunization, the mice that had been pretreated in the *absence* of thymocytes (the thymus-deprived mice) could respond almost as well as non-pretreated controls. On the other hand, mice pretreated in the *presence* of thymocytes (the thymus-reconstituted mice) were totally unable to respond to an immunizing dose of antigen even after the addition of further thymocytes. Thus the presence of thymus-derived lymphocytes during the course of the tolerance induction had created a milieu in the experimental animal where normal thymocytes could not co-operate with pretreated bone-marrow-derived cells (BMDC).

To gain further insight into how the co-operation of these two cell types had been abrogated, we have transferred the spleen cells of pretreated mice into thymectomized lethally irradiated recipients to see if they can co-operate with normal thymocytes in a new environment. This manoeuvre removes the cells from a possible source of residual antigen or other potential immunosuppressive factors that might be circulating in the original host.

We have found that not only will the spleen cells from animals pretreated in the presence of thymocytes not co-operate with normal thymocytes in the new environment, but that they will also prevent the normal thymocytes from co-operating with normal BMDC.

MATERIALS AND METHODS

Mice

Male or female CBA mice were used in these experiments. They were of either strain CBA/H T_6T_6 from our own colony or strain CBA/J from Jackson Laboratories. All experiments were controlled for sex or strain of mouse.

Thymectomy

Thymectomies were performed on adult mice, 7–8 weeks of age, under light ether anaesthesia following the technique of Miller (1960). At the termination of experiments all mice were autopsied and thymic remnants were searched for. None were found in any animals used in these experiments.

Irradiation

A mid-axis dose of 850 R was delivered from a Siemans 250 kV machine at a rate of 85 rad/min.

Cell suspensions

Bone marrow cell suspensions were prepared by washing out the femurs of adult syngeneic mice with cold sterile medium M199. Thymus cell suspensions were prepared by gently teasing thymus glands of syngeneic weanling (4–5 weeks of age) mice between sterile glass slides in cold M199, filtered through gauze, and washed before injection. Spleen cell suspensions were made by the same technique. Counts of viable cells were made in a haemocytometer using the trypan blue dye exclusion method. The cells were inoculated intravenously via the tail vein.

Red blood cells

These were obtained in Alservers solution and washed three times in saline before use.

Bleeding

Blood was collected from the retro-orbital sinus by capillary pipette. Serum was separated and used for titration within 24 hours. Individual mice were ear-marked so that each could be followed serially.

Titrations

All sera were individually titrated by the microhaemagglutination technique of Sever

(1962). Titres are expressed as the $\log_2$ of the last well showing macroscopic agglutination. After the results were recorded, the red cells were resuspended by gentle tapping of the plates and 0·025 ml of 0·1 M 2-mercaptoethanol (ME) was added to each well. The cells were allowed to resettle at room temperature and end-points were read as before. These titres were taken to represent ME resistant (MER) antibody. This method of ME inactivation has been studied at some length and has been shown to produce the same results as more standard techniques (Scott and Gershon, 1970). It was used in these studies in order to minimize the blood loss of experimental animals. MER antibody, although not exactly analogous, is roughly equivalent to 7S antibody under ordinary circumstances (Adler, 1965).

Statistical analysis

Student's *t*-test was used in all statistical analyses.

EXPERIMENTAL PLAN

An outline of the protocol followed is presented in Fig. 1. The method used to induce tolerance was the same as the one previously reported (Gershon and Kondo, 1970). Four days after the termination of tolerance induction, the spleen cells of the treated and control mice were transferred to thymectomized mice that had been lethally irradiated and bone marrow grafted 30 days previously. Some of these secondary recipients were given additional normal thymocytes. The resultant chimeras were then immunized with either a 20 per cent suspension of SRBC, a 20 per cent suspension of horse red blood cells (HRBC) or were unimmunized.

The numbers of thymocytes and spleen cells that were given varied from experiment to experiment, and the numbers for each individual experiment are given in the text.

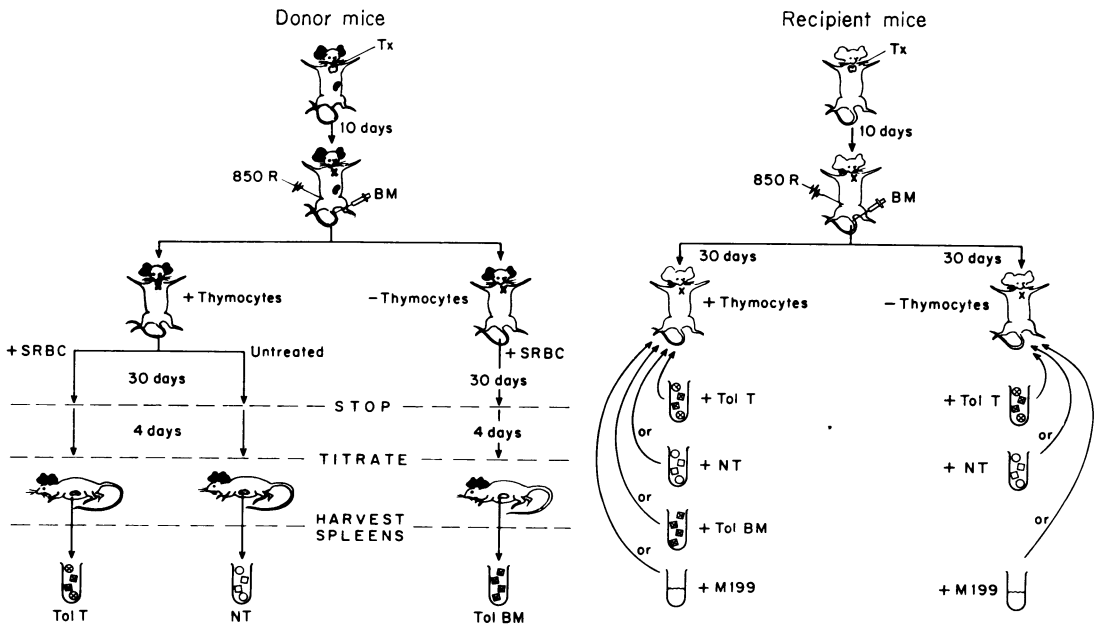

FIG. 1. Outline of Experimental Plan.

RESULTS

The first experiment we report was performed on CBA/HT_6T_6 mice. The donor mice had been reconstituted with 3×10^7 thymocytes and after the termination of tolerance induction 1×10^8 of their spleen cells were transferred to thymus-deprived recipients. Some of these recipients also received an inoculation of $3{\cdot}0 \times 10^7$ normal thymocytes.

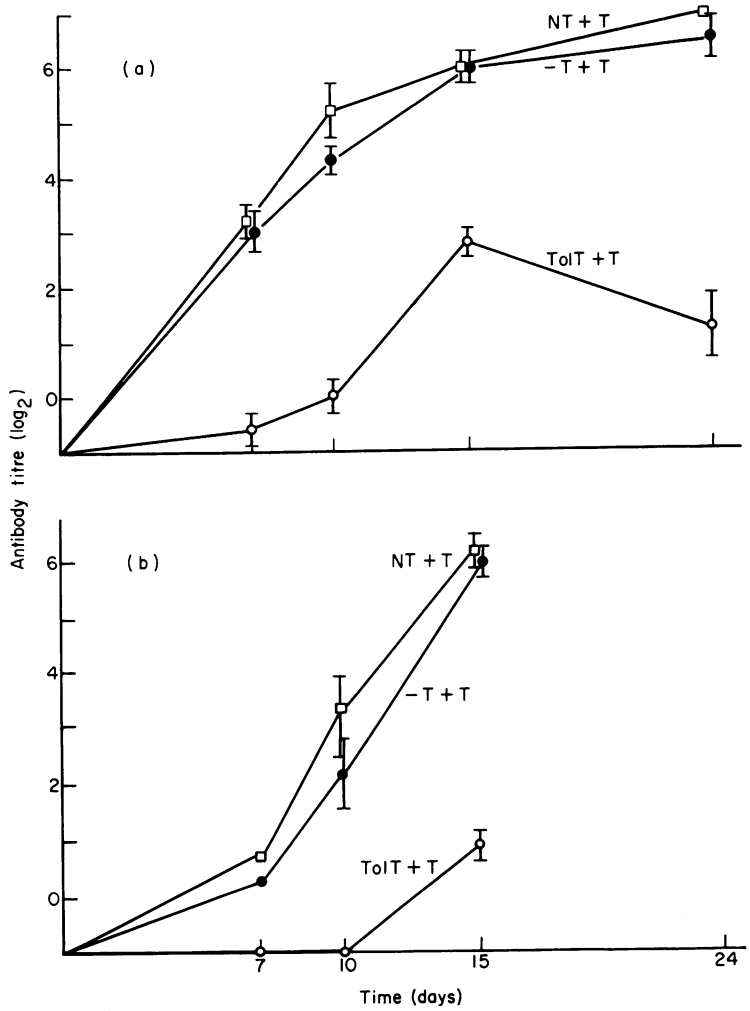

FIG. 2. Anti-SRBC response (±S.E.) of thymus-deprived mice given spleen cells from thymus-reconstituted chimeras (see Table 1) and normal thymocytes. (a), Total antibody. (b), MER antibody. (NT+T), Donor mice untreated; normal thymocytes added; (−T+T), no donor cells given; normal thymocytes added; (TolT+T), donor mice given SRBC; normal thymocytes added.

In Fig. 2 the anti-SRBC response of three groups of recipient mice, all of which got normal thymocytes, is given. In addition to the normal thymocytes, one group received spleen cells from untreated mice (NT), one received no spleen cells (−T), and one

received tolerant spleen cells (TolT). Two points are clearly made: (1) The addition of untreated spleen cells did not significantly affect the immune response (NT *vs* −T).* (2) The addition of tolerant spleen cells significantly decreased the response (TolT *vs* NT or −T). The inhibition produced by the tolerant spleen cells was much more marked in the MER fraction of antibody (ME inactivation was not performed on the anti-serum on day 24).

Fig. 3 gives the anti-SRBC titres of recipient mice that got the same spleen cells as the mice presented in Fig. 2, without the addition of normal thymocytes. It is clear that the

TABLE 1

PROBABILITY ($P<$) ANALYSES OF THE DATA PRESENTED. SEE VARIOUS FIGURES FOR EXPLANATIONS OF GROUPS

			Day				
			5	7	10	14 or 15	21 or 24
FIG. 2.	NT+T	Total		0·001	0·001	0·001	0·001
	vs						
	TolT+T	MER		0·02	0·001	0·001	
	−T+T	Total		0·001	0·001	0·001	0·001
	vs						
	TolT+T	MER		0·05	0·001	0·001	
FIG. 2.	TolT+T						
vs	*vs*	Total		N.S.	N.S.	N.S.	N.S.
FIG. 3.	TolT−T	MER*		N.S.	N.S.	N.S.	
	NT+T	Total		0·001	0·001	0·001	0·001
	vs						
	NT−T	MER*		0·05	0·001	0·001	
	−T+T	Total		0·001	0·001	0·001	0·001
	vs						
	−T−T	MER*		0·05	0·01	0·001	
FIG. 4.	TolT+T	Total		0·05	0·02	0·02	0·001
(HRBC)	*vs*						
	TolT−T	MER*		N.S.	0·05	0·05	
FIG. 5.	TolT+T	Total		N.S.	0·05	N.S.	0·02
	vs						
	TolBM+T	MER		N.S.	N.S.	0·001	0·01
FIG. 6.	TolT+T	Total	N.S.	0·5	0·001	0·001	
	vs						
	−T+T	MER	N.S.	N.S.	0·01	0·001	
	TMMT+T	Total	N.S.	0·001	0·001	0·01	
	vs						
	−T+T	MER	N.S.	0·001	0·001	0·01	
FIG. 7.	IMMT (high)+T	Total	N.S.	N.S.	N.S.	N.S.	
	vs						
	IMMT (low)+T	MER	N.S.	N.S.	N.S.	N.S.	
	IMMT (low)+T	Total	N.S.	N.S.	N.S.	N.S.	
	vs						
	TolT+T	MER	N.S.	N.S.	N.S.	N.S.	
	IMMT (high)+T	Total	N.S.	N.S.	0·01	0·01	
	vs						
	TolT+T	MER	N.S.	N.S.	0·02	0·001	

* Titres not given in figures.

* Table 1 summarizes the *P* values for all the experiments reported herein.

spleen cells of untreated donors did not contain enough thymus-derived lymphocytes to augment the anti-SRBC response (NT *vs* −T). A comparison of Fig. 4 with Fig. 3 reveals that the addition of thymocytes to the recipients significantly augmented their response if they had been given normal donor spleen cells (NT) or no donor spleen cells (−T), but did not if they had been given tolerant spleen cells (TolT).

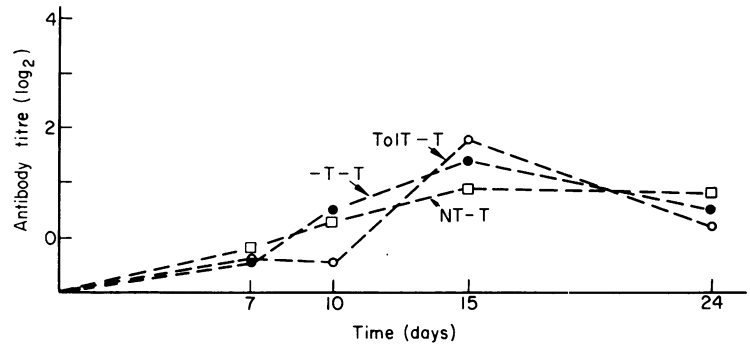

FIG. 3. Anti-SRBC response of thymus-deprived mice given spleen cells from thymus-reconstituted chimeras (see Table 1); no additional normal thymocytes were given. (a) Total Antibody. (NT−T), Donor mice not given SRBC; normal thymocytes not added; (−T−T), no donor cells given; normal thymocytes not added; (TolT−T), donor mice given SRBC; normal thymocytes not added.

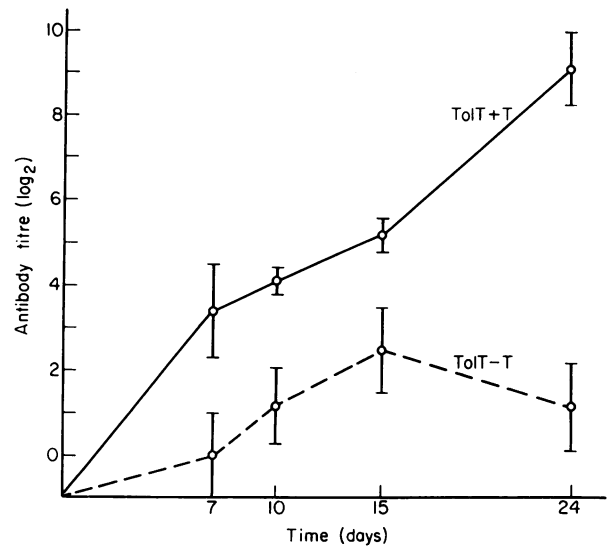

FIG. 4. Anti-HRBC response (±S.E.) of thymus-deprived mice given spleen cells from thymus-reconstituted chimeras (see Table 1); some mice also got normal thymocytes. (a) Total antibody. (TolT+T), Donor mice given SRBC; normal thymocytes added; (TolT−T), donor mice given SRBC; normal thymocytes not added.

A specificity control from this experiment is presented in Fig. 4. It can be seen that the addition of tolerant spleen cells did not prevent thymocytes from augmenting the immune response to HRBC.

Another experiment was done to test whether thymocytes had to be present in the donor spleen for the immunosuppressive effect to occur. In this experiment half the donors were

given 3×10^7 thymocytes on the day of irradiation and bone marrow reconstitution. All animals were then given the standard SRBC pretreatment and 4 days after the last injection of SRBC 1×10^8 spleen cells plus 3×10^7 normal thymocytes were transferred to thymus-deprived recipients. This experiment was also done on T_6 mice.

Fig. 5 demonstrates that only the spleen cells of thymus-reconstituted mice produced an immunodepression after adoptive transfer. As in previous experiments, the suppression was most marked in the MER fraction of antibody.

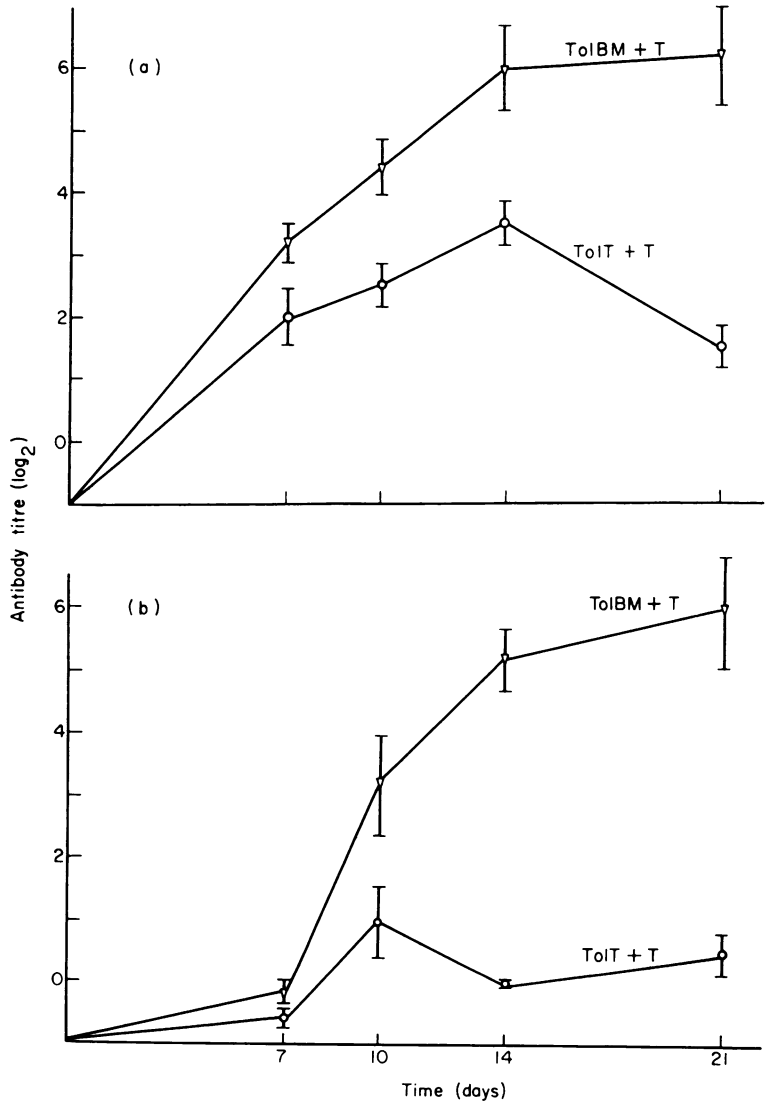

FIG. 5. Anti-SRBC response (± S.E.) of thymus-deprived mice given spleen cells from thymus-deprived chimeras (see Table 1) some of which had been reconstituted with thymocytes. All donor mice were pretreated with SRBC. (a) Total antibody. (b) MER antibody. (Tol BM+T), Donor mice not reconstituted with thymocytes; normal thymocytes added; (TolT+T), donor mice reconstituted with thymocytes; normal thymocytes added.

The experiments presented above show that spleen cells from thymus-reconstituted mice that have been pretreated with large amounts of SRBC can prevent normal thymocytes and BMDC from co-operating in a secondary recipient. We present two more experiments below which confirm these findings and which also show the difference in the effect the adoptively transferred spleen cells may have dependent upon whether or not the donor mice make antibody during the pretreatment.

In the first experiment twenty-four CBA/J mice were pretreated after reconstitution with 4×10^7 thymocytes. At the termination of pretreatment eleven mice had antibody titres of $\log_2$ 1 or less. These antibodies were all ME sensitive. Ten mice made antibody with a titre of $\log_2$ 6 or more, which was mostly MER (mean antibody titre $\log_2$; total 6·7; MER 6·2). Separate spleen cell suspensions were made from these two groups of donor mice and 8×10^7 cells were given to thymus-deprived chimeras along with 3×10^7 normal thymocytes. The recipients were then immunized with SRBC.

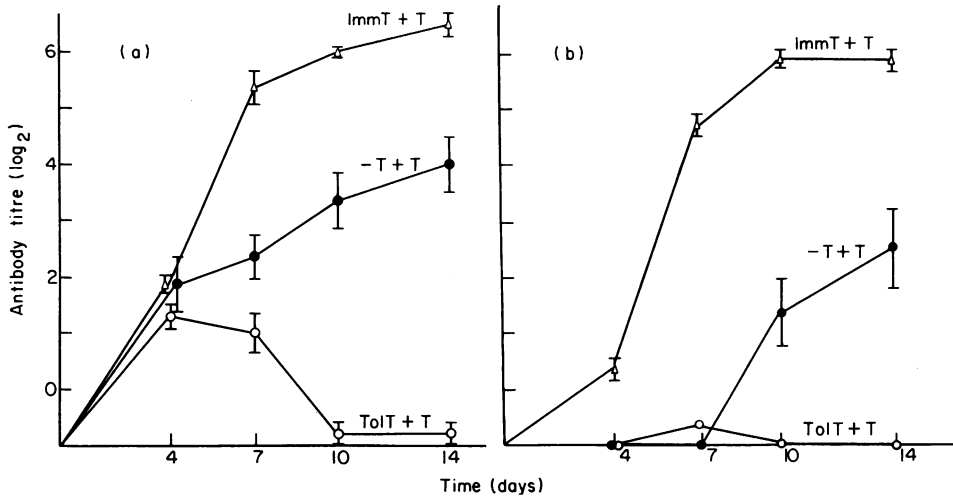

FIG. 6. Anti-SRBC response (±S.E.) of thymus-deprived mice given spleen cells from thymus-reconstituted chimeras (see Table 1) and normal thymocytes. All donor mice were pretreated with SRBC. (a) Total antibody. (b) MER antibody. (IMMT+T), Donor mice made antibody; normal thymocytes added; (TolT+T), donor mice did not make antibody; normal thymocytes added; (−T+T), no donor cells given; normal thymocytes added.

The results (Fig. 6) show that the spleen cells from the antibody making mice were immune; recipients of these cells made significantly more antibody than controls (IMMT *vs* −T). On the other hand recipients that got spleen cells from the mice that made no antibody made significantly less antibody than controls (TolT *vs* −T). In fact they made no MER antibody at all.

The last experiment we report is one in which more donor mice made antibody than in the previous experiment. These were also CBA/J mice and they had been reconstituted with $1{\cdot}5 \times 10^7$ thymocytes prior to the antigen pretreatment. Five donor mice made no antibody at all, five made antibody with a $\log_2$ titre between 4 and 5 (total 4·8; MER 4·6) and twenty-seven made antibody with a $\log_2$ titre between 6 and 8 (total 7·7; MER 7·1). Twelve $\times 10^6$ spleen cells from three separate pools were given to thymus-deprived chimeras along with 3×10^7 normal thymocytes.

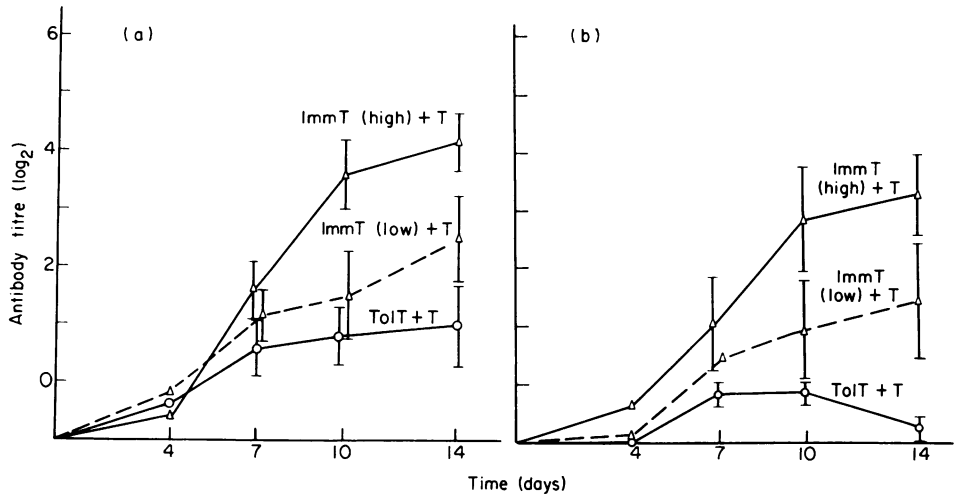

FIG. 7. Anti-SRBC response (±S.E.) of thymus-deprived mice given spleen cells from thymus-reconstituted chimeras (see Table 1) and normal thymocytes. All donor mice were pretreated with SRBC. (a) Total antibody. (b) MER antibody. (IMMT (high)+T), Donor mice made titres > log_2 6; normal thymocytes added; (IMMT (low)+T), donor mice made titres between log_2 4–5; normal thymocytes added; (TolT+T), donor mice did not make antibody; normal thymocytes added.

The results (Fig. 7) show that the more antibody the donor mice had made, the more antibody the recipients of their spleen cells made.

DISCUSSION

The results presented above demonstrate that the adoptive transfer of spleen cells from mice made tolerant to SRBC clearly and specifically prevents the co-operation of normal thymocytes and normal BMDC. The results also demonstrate that it is necessary for thymus-derived lymphocytes to be present during the course of tolerance induction for this phenomenon to occur.

We have considered three general mechanisms by which the adoptive transfer of tolerance may be produced. They are: (1) The transfer of antigen, (2) The production of an immunosuppressive substance by the transferred BMDC and (3) The production of an immunosuppressive substance by the transferred thymus-derived cells.

1. *The transfer of free antigen.* This mechanism seems least likely to us since the adoptive transfer of spleen cells from mice treated in the absence of thymus-derived lymphocytes did not produce adoptive tolerance. Thus in order to postulate that antigen was a causative factor one would have to postulate that it was thymus 'processed' antigen, which was particularly tolerogenic.

2. *The effect is caused by a product of the BMDC.* The most well known product of BMDC is antibody (Davies, 1969; Miller and Mitchell, 1969) and it is well established that antibody can interfere with the immune response (see Uhr and Moller, 1968). For the following reasons we think that it is unlikely that conventional antibody is responsible for the effect we have reported. (a) Suppression occurred when neither donor mice nor their transferred spleen cells made any significant amount of detectable antibody. (b) Transferred spleen cells from donor mice that did make antibody did not produce a shut-off effect. The possibility that some exhaustively differentiated antibody making cells (Sterzl, 1966),

released a small amount of antibody and then went no further seems unlikely to us as the donor mice had large pools of antibody-making precursor cells and only small numbers of thymus-derived lymphocytes to activate them. (c) The ability of antibody to interfere with antibody production is related to the affinity of the antibody, with high affinity antibodies being most efficient (Walker and Siskind, 1968). Partially tolerant animals make antibody of low affinity (Theis and Siskind, 1968). Thus it seems unlikely that unmeasurable amounts of low affinity antibody could cause this effect. (d) In the experiments reported above there was always a preferential effect on the MER fraction of antibody. Passive antibody has not been reported to have this effect. Indeed it appears that the shut-off ability of passive antibody may preferentially affect ME sensitive antibodies (Sahiar and Schwartz, 1964; Wigzell, 1966; Morris and Moller, 1968; Uhr and Moller, 1968). There appears to be no more basis for ascribing the effects we have reported to the production of conventional antibodies than for considering this to be the mechanism by which tolerance is generally produced.

3. The shut-off effect is produced by a product of the thymus-derived lymphocytes. By exclusion this mechanism appears most likely to us. The product might be either directly produced by the thymus-derived lymphocytes in the transferred spleens or indirectly produced by the BMDC that have been influenced by them. We hope to be able to distinguish between these two possibilities with the use of an anti-theta anti-serum (Raff, 1969).

The immuno-enhancing effect of thymus-derived lymphocytes has been ascribed to a putative immunoglobulin called IgX (Mitchison, 1968). Following simple algebra, we suggest that the putative immunosuppressive substance be called IgY (Gershon and Kondo, 1970).

There are a number of disparate observations in the literature, some of which are difficult to explain otherwise, which could all be explained or united by an IgY hypothesis. Most striking of these is the observation that *in vitro* incubation, a procedure that kills large lymphocytes, abrogated the tolerance of a population of thoracic duct cells (McGregor, McCullagh and Gowans, 1967). We would theorize that the cells killed were the IgY producing thymus-derived lymphocytes which were responsible for the tolerance.

A number of workers have observed that the adoptive transfer of normal syngeneic immunocompetent cells into tolerant animals does not abrogate the tolerant state (Chase, 1963; Crowle and Hu, 1969; Tong and Boose, 1970; McCullagh, 1970). McCullagh also showed that the transferred cells became unresponsive 3 days after transfer into the tolerant hosts. Although the possibility that residual antigen rendered the adoptively transferred cells tolerant was never completely excluded, the observation by Tong and Boose that even immunized cells were unable to break tolerance, renders this explanation unlikely in our opinion.

Horiuchi and Waksman (1968) injected antigen into the thymuses of normal adult rats and showed that significant amounts of antigen did not escape from the thymus into the circulation. This procedure rapidly rendered the rats partially tolerant. The conclusion that the antigen injected rendered the cells in the thymus tolerant cannot explain the results entirely. Rendering thymus cells tolerant should have no immediate effect on the immunocompetence of an adult animal, unless the tolerance were infectious, as thymectomy itself at that age does not affect the immune response.

Baker, Stashak, Amsbaugh, Prescott and Barth (1970) have noted that thymectomy and ALS treatment increases the immune response of mice to pneumococcal polysaccharide,

again suggesting the thymus-derived lymphocyte might make a product which shuts off other cells. Our observation that thymus-derived cells shut-off other cells in antigenic competition is also in line with this idea (Gershon and Kondo, 1971).

An IgY would be of great intellectual comfort in explaining ultra low zone tolerance (Shellam and Nossal, 1968). The amount of antigen used to produce tolerance in this situation can hardly be explained without resorting to some mechanism of amplification.

The fact that immunosuppressive agents can prevent tolerance induction (Claman and Bronsky, 1968) suggests that tolerance is an active process such as might be required for the production of IgY.

Last and perhaps most direct is the observation that the adoptive transfer of antigen pretreated lymphoid cells can abrogate the delayed hypersensitivity response of recipient mice immunized to that antigen (Crowle and Hu, 1969). These authors have suggested that the lymphoid cells make a substance they call 'contrasensitizer†.'

For the above stated reasons we favour the hypothesis that thymus-derived lymphocytes make an immunosuppressive substance, to explain the results we have presented. We would like to emphasize that at the present time we consider this a working hypothesis and that more information is needed before alternate explanations can be ruled out.

If our results do nothing else they vitiate one of the interpretations we have previously made (Gershon and Kondo, 1970). We had favoured the interpretation that the inability of thymocytes to break tolerance was due to tolerance of the BMDC. Since it now appears that the tolerance in our system can be spread from cell to cell this conclusion cannot be validated by our data. Nonetheless, the recent results of Playfair (1969) and of Chiller, Habicht and Weigle (1970) showing specific unresponsiveness of bone marow cells from tolerant animals, are less likely to have been caused by IgY producing thymus-derived lymphocytes. Nonetheless, a remote possibility that the bone marrow cells were contaminated with small numbers of thymus-derived lymphocytes must be considered. We estimate that no more than several hundred thousand thymus-derived lymphocytes were present in the spleen cell suspensions we transferred. It is possible that very few contaminating cells could produce the effect.

Lastly, we would like to comment on why we have been able to adoptively transfer tolerance while some other workers have not. We believe this is because we transferred cells to minimally reconstituted animals wherein the effect of a small amount of IgY production could be seen. When we transferred spleen cells that could produce an effect in reconstituted chimeras to normal animals, we failed to observe an immunosuppressive effect (Gershon and Kondo, unpublished observations). That the effect is small and difficult to see does not necessarily mean, however, that the same is true for its significance.

ACKNOWLEDGMENTS

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† More recently several new reports of adoptive tolerance production in immunocompetent mice have appeared. (Asherson, G. L., Zembala, M. and Barnes, R. M. (1971) *Clin. exp. Immunology* **9**, 109.) (Terman, D. S., Minden, P. and Crowle, A. J. [1971]. 'Adoptive transfer of neonatal tolerance into normal mice.' *Fed. Proc.*, **30**, 650.)

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