Immunology 1978 **34** 167

Depression of cell-mediated immunity following inoculation of *Trichinella spiralis* extract in the mouse

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Received 23 *December* 1976; *accepted for publication* 10 *March* 1977

Summary. Mice pretreated with *Trichinella spiralis* extract (TsE), or infected with the parasite, rejected primary skin allografts in 18–23 days and secondary allografts in 12–16 days. Mice pretreated with saline or with bovine serum albumin (BSA) rejected the primary allografts in 12–18 days and did not accept the secondary grafts.

Inoculation of increasing doses of parental spleen cells from mice pretreated with saline or with BSA in F_1 hybrids produced proportionately stronger graft-versus-host reactions (GvHR) whereas increasing doses of cells from TsE pretreated mice reduced proportionately the capacity of the inoculum to induce a GvHR. Immunodepression of the parental cells was obtained with 7 and with 4, but not with 2, daily injections of TsE. The depression waned rapidly after the treatment with TsE but a significant degree still remained after 3 days.

Immunodepression by TsE cannot be solely explained by antigenic competition and although our results are consistent with the induction of suppressor cells, it is probable that other mechanisms are also involved.

INTRODUCTION

Recent reports on immunodepression elicited by parasitic infections (see Ciba Foundation, 1974) have contributed to the elucidation of the apparent paradox of parasites persisting in seemingly immunocompetent hosts. Svet-Moldavsky, Shaghijan, Chernyahovskaya, Mkheidze, Litovchenko, Ozeretkovskaya & Kadaghidze (1970) observed that *Trichinella spiralis* infected mice retained primary and secondary skin allografts considerably longer than non-infected mice. They did not determine, however, whether the reduced responsiveness was a specific effect caused by the parasite, the result of antigenic competition by parasite antigens, or the consequence of a general physiological deterioration in the diseased animals.

Faubert & Tanner (1971) found that massive infections or inoculations of serum from infected mice or rabbits reduced the antibody response to sheep red blood cells (SRBC) in mice. A similar effect, however, was attained by injection of normal rabbit serum which was attributed to antigenic competition (Tanner, personal communication). Cypess, Lubiniecki & Hammond (1973) reported a depressed humoral response to Japanese B encephalitis virus in *T. spiralis* infected mice but later (Lubiniecki, Cypess & Lucas, 1974) they found that *T. spiralis* infections did not affect the number of anti-SRBC plaque forming cells or the level of anti-SRBC antibodies produced by mice although the sequestration of the sheep cells by the liver and the spleen was altered. In this latter experiment, mice were immunized with SRBC on the 7th day of infection when the parasitemia and the stimulation of the

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lymphoid system is negligible (Harley & Gallichio, 1971; Ruitenberg & Duyzings, 1972; Faubert & Tanner, 1974a) and, therefore, it might have been too early for the parasite to elicit an effective immunodepression. This notion is supported by Faubert (1976) who found that infected mice had a reduced ability to produce plaque forming cells on days 14 and 28 post infection but not on days 7 or 56.

Faubert & Tanner (1974b) reported that bone marrow cells from infected mice were inefficient in reconstituting anti-SRBC responsiveness in thymectomized, irradiated mice and that a soluble fraction of *T. spiralis* was agglutinating and cytotoxic for mouse lymph node cells (Faubert & Tanner, 1975). Barriga (1975) observed that infection with *T. spiralis* or inoculation of extracts reduced the capacity of mice to form antibodies to the T-cell dependent antigen SRBC but not to the T-cell independent antigen polyvinylpyrrolidone (PVP).

The results of Lubiniecki, Cypess & Lucas (1974) indicate some alteration of the macrophage function in infected animals but since they could not detect immunodepression in their mice, it is questionable whether modifications of the macrophage activity are related to the reduction of the humoral response observed by others. Furthermore, transfer of bone marrow cells from infected animals to thymectomized and irradiated recipients failed to reconstitute the anti-SRBC response (Faubert & Tanner, 1974b) although the macrophages in the recipients should not have been affected by this treatment (Unanue, 1972). On the other hand, the reduced response to allografts observed by Svet-Moldavsky *et al.* (1970) and the failure to depress antibody formation to PVP found by Barriga (1975) strongly suggest that the immunodepressive effect by *T. spiralis* affects mainly the T-cell compartment.

In this paper we attempt to verify the effect of diverse treatments with *T. spiralis* extracts on typical T-cell responses like allograft rejection and graft versus host reaction (GvHR) and draw some conclusions about its most probable mechanism of action.

MATERIALS AND METHODS

Animals, parasite, and inoculations

Female C57Bl/6J mice were utilized as skin graft recipients and as spleen cell donors. Female AKR mice were used as skin graft donors and female B6D2F$_1$ (C57Bl/6J mothers × DBA/2J fathers) hybrid mice as spleen cell recipients. All animals were purchased from The Jackson Laboratories, Bar Harbor, Maine.

The strain of *T. spiralis* utilized was originally obtained from Dr N. F. Weatherly, School of Public Health, University of North Carolina, and maintained in our laboratory for 3 years by passage in Swiss mice. The infections were done by oesophageal inoculation of washed larvae and the inoculations by intraperitoneal injections in the lower-right quadrant of the abdomen. The extracts of *T. spiralis* were obtained by pepsin-HCl digestion of carcasses infected 30–40 days previously, repeated washes of the freed larvae in 0·15 M NaCl (saline), 1 wash in 10% petroleum ether in saline (vol/vol) followed by 3 washes in saline and homogenization of the clean larvae in 0·075 M NaCl at 45,000 rpm for 10 periods of 1 min each followed by 3 15-s bursts of sonication. The homogenate was clarified by centrifugation at 16,000 *g* for 30 min and the supernatant fluid was stored at −20° until used as *T. spiralis* extract (TsE). The entire procedure was carried out at 2° or in an ice-bath. Enumeration of the antigens in the extract was done by immunoelectrophoresis with serum of a rabbit hyperimmunized with the same extract.

Foetal calf serum (FCS; Grand Island Biological Co., Grand Island, New York) was inactivated at 56° for 30 min, absorbed in 25% SRBC (vol/vol), and stored at −20° until used as control for antigenic competition in some experiments. Crystallized bovine serum albumin (BSA; Pentax, Kankakee, Illinois) was used for antigenic competition in other experiments and as a standard to adjust the protein content of the TsE and the FCS at 1 mg/ml by the biuret technique.

Infections were done with 45 viable larvae and inoculations with 0·2 mg of protein of the respective preparation, per mouse.

Spleen cells were obtained by killing the donor mice by cervical dislocation, collecting their spleens immediately, and dissociating the cells by pushing the finely minced organ through a 64-gauge nylon mesh into a dish with RPMI-1640 medium without addition of protein. The cells were washed by centrifugation at 400 *g* for 10 min in the same medium, resuspended in 0·17 M ammonium chloride in Tris buffer pH 7·2 to lyse the erythrocytes, and washed 3 more times in medium. The cell concentration was estimated with a Fisher Autocytometer II and

viability was determined by trypan blue exclusion tests. All the procedures were carried out in sterile environment and at 2° or in an ice-bath.

For the GvHR experiments, the relative weight of the spleen of each recipient mouse was expressed as mg/10 g of body weight and these figures were utilized to obtain the mean and the standard deviation for each group. The significance of the difference between each experimental group and the appropriate controls was determined by the Student's *t*-test. The spleen indices were expressed as the anti-log of the difference between the mean $\log_{10}$ of the experimental and the control groups.

RESULTS

Experiment 1

Three groups of 4 C57Bl/6J mice each received daily inoculations of saline, BSA and TsE, respectively for 29 days and a 4th group was infected (S-, BSA-, TsE- and infected mice, respectively). On the 2nd day of inoculation and on the 7th day of infection, all the mice were anaesthetized with Fluothane (Ayerst Lab, Inc., New York) and grafted with about 1 cm² of skin of AKR mice on the right costal wall. The bandages were removed on the 6th day and the grafts were observed daily for 29 days. The condition of the grafts and the surrounding area was recorded as 4 conventional grades: 1X (mild inflammation), 2X (intense inflammation), 3X (evidence of necrosis), and 4X (sloughing). Identical grafts were placed in the left costal area of the same animals 7 days after suspending the inoculations and observed for 16 days.

S-mice showed a mild inflammation around the graft on the 9th day which progressed steadily to sloughing of the graft in one mouse on the 12th day, in 2 mice on the 13th day and in the remaining mouse on the 16th day (av. 13·5 days). BSA-mice revealed mild inflammation on the 9th and 10th day with steady progress to sloughing in one mouse on the 12th day, in one mouse on the 14th day, in another mouse on the 16th day and in the remaining animal on the 18th day (av. 15 days).

Infected mice showed mild inflammation on the 9th day that remained the same until the 13th day. From the 14th to the 18th day the inflammation intensified and there was some evidence of necrosis on days 18 and 19 but actual sloughing was never observed. After day 19 the inflammation receded slowly and was almost unnoticeable on day 29.

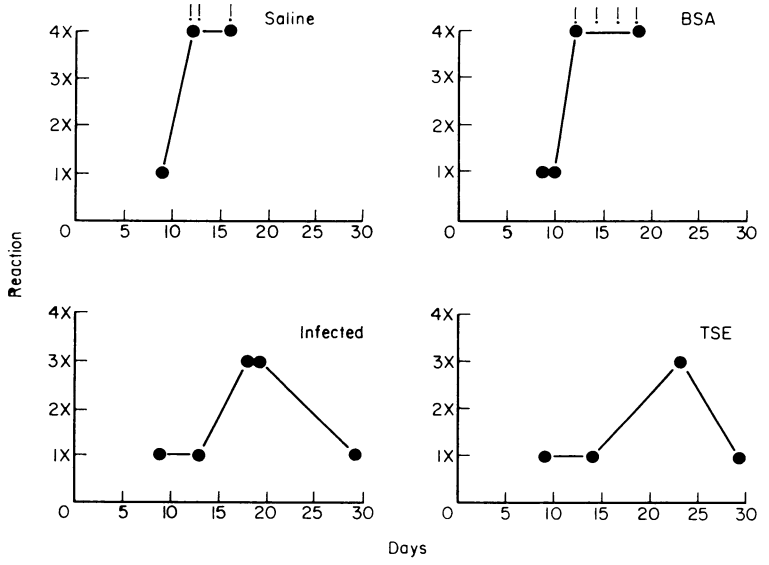

Figure 1. Evolution of primary AKR skin allografts in C57Bl/6J mice inoculated with 0·15 M NaCl (saline), bovine serum albumin (BSA) or *T. spiralis* extract (TsE) or infected with 45 viable *T. spiralis* larvae (infected). 1X = mild inflammation; 2X = intense inflammation; 3X = necrosis; 4X = sloughing; ! indicates when sloughing was observed.

Table 1. Spleen indices of F_1 hybrid mice receiving splenocytes from parental mice pretreated with 0·15 M NaCl (saline), bovine serum albumin (BSA), or *Trichinella spiralis* extract (TsE)

Group	No. mice	Treatment	Cell dose	Spleen index	*P*
S_3	10	Saline	3×10^6	1·000	Base
BSA_3	9	BSA	3×10^6	1·321	<0·001
TsE_3	9	TsE	3×10^6	0·939	>0·30
S_7	6	Saline	7×10^6	1·000	Base
BSA_7	6	BSA	7×10^6	1·130	≃0·20
TsE_7	6	TsE	7×10^6	0·647	≃0·05
S_{20}	10	Saline	20×10^6	1·000	Base
BSA_{20}	9	BSA	20×10^6	0·991	>0·20
TsE_{20}	9	TsE	20×10^6	0·587	<0·001

Table 2. Spleen indices of F_1 hybrid mice receiving 10^7 splenocytes from parental mice pretreated with 0·15 M NaCl (saline), foetal calf serum (FCS), or *Trichinella spiralis* extract (TsE) for 2 or for 4 days, or receiving no cells

Group	No. mice	Treatment (days)	Spleen index	*P*
N	8	None	1·000	Base
S_2	7	Saline (2)	2·545	<0·001
FCS_2	9	FCS (2)	1·672	<0·005
TsE_2	10	TsE (2)	1·659	<0·005
S_4	8	Saline (4)	1·826	<0·001
FCS_4	8	FCS (4)	1·868	<0·001
TsE_4	9	TsE (4)	1·110	>0·10

TsE mice revealed mild inflammation between the 9th and the 14th day which intensified somewhat up to the 19th day. The only evidence of necrosis was seen on day 23 and on day 29 the area looked almost normal again (Fig. 1).

Secondary grafts placed on the same mice did not take in S- and BSA-mice but healed and remained *in situ* for 12 and for 16 days respectively in infected and TsE-animals.

Experiment 2

Sixty-six C57Bl/6J mice, about 20-weeks old, were divided into 3 groups and injected daily for 7 days with saline, BSA and TsE, respectively (groups S-, BSA-, and TsE-donor mice). On day 1 after the last inoculation their spleen cells were collected separately and inoculated into 3 groups of 8–15-week-old F_1 hybrid mice (groups S-, BSA-, and TsE-recipient mice). Each of these groups were further divided into 3 sub-groups, each receiving 3×10^6, 7×10^6 and 20×10^6 of the respective cells. The recipients were killed and their spleen indices calculated on the 9th day after cell transfer.

Inoculation of 3×10^6 spleen cells from TsE-mice caused almost identical spleen reaction as inoculation of the same number of cells from S-donors. Inoculation of cells from BSA-mice, however, induced a significantly greater reaction (Table 1).

Inoculation of 7×10^6 cells from TsE-mice resulted in a reduced spleen reaction as compared with the controls, the difference being on the borderline of what is conventionally considered statistically significant. The spleen enlargement induced by inoculation of cells from BSA-donors was somewhat greater than the saline controls but not statistically significant.

Inoculation of 20×10^6 cells from TsE-donors resulted in a greatly reduced spleen reaction as compared with the GvHR produced by S- and BSA-cells which were almost identical.

Experiment 3

Thirty 20-week old C57Bl/6J mice were divided into 3 equal groups and each of these received inoculations of saline, FCS or TsE. Half of the mice received 2 daily inoculations and the other half received 4 daily inoculations. One day after the last inoculation their spleens were retrieved and 10×10^6 cells were inoculated into 6 groups of F_1 hybrids, about 5 weeks of age, in such a manner that each group received cells from donors treated with saline, BSA or TsE for 2 or for 4 days.

On the 9th day after the transfer the recipients were killed and their spleen indices determined. Eight F_1 hybrids which had not been inoculated with cells (normal hybrids) were killed on the same occasion to provide a base of comparison with the spleens of normal mice.

Inoculation of 10×10^6 spleen cells from S-, FCS- or TsE-donors pretreated for 2 days caused an intense and significant GvHR as compared with normal hybrids, the enlargement of the spleen being considerably greater, however, in those recipients transferred with S-cells (Table 2).

Inoculation of cells from S- or FCS-donors pretreated for 4 days still induced a marked spleen reaction which was almost identical in both groups. The reaction caused by FCS-cells from animals

Table 3. Spleen indices of F_1 hybrid mice receiving 10^7 splenocytes from parental mice pretreated with 0·15 M NaCl (saline), foetal calf serum (FCS), or *Trichinella spiralis* extract (TsE) and collected 2 or 3 days after the end of the treatment, or receiving no cells

Group	No. mice	Treatment	Day of transfer	Spleen index	*P*
N	7	None	—	1·000	Base
S_{II}	7	Saline	2nd	2·147	<0·001
FCS_{II}	5	FCS	2nd	1·850	<0·001
TsE_{II}	7	TsE	2nd	1·624	<0·001
S_{III}	6	Saline	3rd	2·116	<0·001
FCS_{III}	6	FCS	3rd	1·723	<0·001
TsE_{III}	7	TsE	3rd	1·352	<0·001

treated for 4 days was in the same range than that caused by FCS-cells from mice treated for 2 days but the S-cells from animals treated for 4 days had a considerably reduced ability to produce a spleen reaction as compared to similar cells from mice treated for only 2 days. Inoculation of TsE-cells from mice treated for 4 days caused a minimum of spleen reaction that statistically was indistinguishable from the spleens of normal hybrids.

Experiment 4

Thirty 7-week old C57Bl/6J mice were divided into 3 equal groups and inoculated daily for 4 days with saline, BSA or TsE. The spleen cells of half of each group were collected on the 2nd day after the last injection and the cells of the other halves on the 3rd day. Ten million cells were transferred to 6 groups of F_1 hybrids, about 6-weeks old, in such a way that each group received cells from donors treated with saline, BSA or TsE until either 2 or 3 days before the transfer. The spleen indices of the recipients and of 7 identical mice which had received no cells were calculated 9 days after the transfers.

Inoculation of 10×10^6 cells from S-, FCS- or TsE-donors collected on the 2nd day after the last injection induced GvHR in all the recipients, as compared to normal hybrids (Table 3). The reaction produced by TsE-cells, however, was considerably weaker than the reactions caused by S- or FCS-cells: although the difference with FCS-cells was not statistically significant, it was suggestive of biological importance.

Cells taken from the donors on the 3rd day after the end of the treatment were also able to induce a GvHR regardless of the treatment but in this case the reaction induced by TsE-cells was significantly smaller than the reactions elicited by S- or FCS-cells.

DISCUSSION

The first-set rejection of skin allografts in S- and BSA-mice followed the well-established pattern in normal mice. Therefore, it can be assumed that these treatments had little, if any, effect on the immune responsiveness of the recipients. The slight delay in the rejection of the grafts in BSA-animals as compared to S-mice may or may not have some significance but it will be considered later in relationship to GvHR. The first-set rejection in infected and in TsE-mice, on the other hand, was considerably delayed as compared with the normal pattern and the S- and BSA-controls. Furthermore, the intensity of the reaction was weaker in the former. Since first-set rejection of allografts is predominantly, if not exclusively, a cell-mediated reaction, these results are a good indication that *T. spiralis* elicits immunodepression by interference with the T-cell function. That a similar amount of BSA did not reproduce the delayed rejection strongly suggests that the reduced responsiveness cannot be explained exclusively by antigenic competition. At the same time, the fact that parasite extracts, in which non-specific toxic activity has not been found (Maeir, Zaiman & Howard, 1962; Gould, 1970), are stronger immunodepressors than actual infections argues against a general deterioration of the physiology of the diseased animals. Furthermore, that large quantities of parasite extract are more immunosuppressive than moderate infections suggests a dose dependent effect.

The delay of the second-set rejection, which can be mediated by a cellular, or a humoral reaction, or both, in infected and in TsE-mice indicates that the parasite or its extract also interferes with the production of memory cells for histocompatibility antigens.

The study of the GvHR induced by inoculation of different numbers of S-, BSA- or TsE-spleen cells yielded some surprising results (Table 1). As expected, the transfer of 3×10^6, 7×10^6 and 20×10^6 cells from S-donors resulted in increasingly marked spleen reactions in the recipients. Inoculation of successively larger number of cells from BSA-donors also showed an increase of the spleen reaction but at a different rate. In fact, injection of 3×10^6 BSA-cells produced a significantly stronger

GvHR than the same number of S-cells: a difference that became smaller and insignificant at larger doses of cells. The reasons for this anomaly deserve further study but two hypotheses can be advanced: Ford, Simmonds & Atkins (1975) found that 4·5–6% of the thoracic duct lymphocytes of unprimed rats could recognize and react to host alloantigens. On the basis of this observation Grebe & Streilein (1976) speculated that there must be more than 10^3 allogenic histocompatibility determinants, or that the receptors of the histocompatibility antigen reactive cells (H-ARC) are only expressed and/or activated in association with other antigen reactive cells. In the first case, it is possible that some cross reactivity exists between BSA determinants and some of the postulated numerous allogeneic histocompatibility determinants in the hybrid mice, in which case the cells from BSA-donors would behave as primed cells against the recipients. In the latter case, the presence of BSA reactive cells would enhance the expression and/or the activation of the H-ARC. Another possibility is that non-specific factors induced by the antigenic BSA, like the blastogenic factor that plays a role in the GvHR (Grebe & Streilein, 1976), would enhance the spleen reaction of the recipients. At higher doses of transferred cells the H-ARC would be in such an excess that any enhancing factor would add little to the final result.

Inoculation of 3×10^6 cells from TsE-donors caused a GvHR similar to that produced by cells from S-donors but injection of 7×10^6 and 20×10^6 TsE-cells showed a progressively reduced ability to stimulate a spleen reaction as compared to the controls. The fact that higher doses of cells exhibited less immunological reactivity indicates the presence of some inhibitory factor that, as found for suppressor cells (Burns, Marrack, Kappler & Janeway, 1975; Thomas, Roberts & Talmage, 1975), requires a threshold concentration to produce a detectable effect. At the same time, it dismisses lymphocytotoxicity as the major mechanism.

Since TsE possesses at least 26 antigenic components according to our immunoelectrophoretic studies (results not shown) whereas BSA is a single molecular species, a more complex mixture of antigens like FCS was selected to evaluate the degree of immudodepression attributable to antigenic competition in our further experiments.

Inoculation of 10×10^6 spleen cells from S-donors treated for 2 days produced an intense GvHR; similar cells from animals treated for 4 days induced a considerable reaction but not as great as the former. This difference may be attributable to partial immunodepression of the donor cells related to the stress of the experimental manipulations, possibly due to the production of endogenous corticosteroids.

Inoculation of cells from FCS-donors treated for either 2 or 4 days induced essentially the same degree of spleen enlargement which was similar to the reaction observed with cells from S-donors treated for 4 days but smaller than the reaction to cells from S-donors treated for 2 days. We took this reduction of the immune competence as the non-specific effect of protein overload, most probably due to the same factors responsible for antigenic competition.

Inoculation of 10×10^6 cells from TsE-donors treated for 2 days elicited the same GvHR as did the cells of FCS-mice treated for 2 days which indicates that any reduction of the immune competence attained by injecting TsE for 2 days may be explained simply by protein overload. Inoculation of similar cells after 4 days of treatment, however, induced virtually no GvHR since the relative weight of the spleen of the recipients was similar to that of hybrids that received no cells (Table 2). The fact that 4 days of treatment were necessary to depress the immune competence of the donor indicates that there must be sustained cellular activity for the depressor factor(s) to act. This observation coincides with reports that suppressor cells appear only after 3–4 days *in vitro* (Burns *et al.*, 1975; Thomas *et al.*, 1975) or *in vivo* (Rich & Rich, 1974; Baker, Stashak, Amsbaugh & Prescott, 1974) systems.

The last experiment revealed that the immunodepressive effect of TsE treatment wanes soon in the spleen after the end of the treatment although it still exists to some degree on the 3rd day. This result is consistent with the findings of Feldman & Kontiainen (1976) who reported that suppressor activity was associated with short-lived lymphocytes and recirculating T cells.

The results reported here indicate that the immunodepression elicited by TsE is stronger than the immunodepression induced by other antigens and therefore suggest that other mechanisms in addition to antigenic competition must operate. The evidence presented points very strongly to the induction of suppressor cells as the major mechanism

of depression. There is a growing consensus among the specialists, however, to consider antigenic competition only as a particular manifestation of a more general homeostatic phenomenon mediated by suppressor cells and directed to set a limit to the clonal expansion occurring during the immune response (Thomas, Roberts & Talmage, 1975). This notion is supported by reports of non-specific immunodepression attributable to suppressor cells induced by BSA, ovalbumin (Ambrose, 1969) and FCS (Burns, Marrack, Kappler & Janeway, 1975).

If this is the case, the differences found between the immune depressive effect of TsE and of BSA and FCS might be rather quantitative than qualitative. Alternatively, mechanisms other than induction of suppressor cells may operate in the TsE system. Although we have not found evidence of lymphocytotoxicity in these experiments and in others, the report by Faubert & Tanner (1975) merits further studies.

The problem of immunodepression by *T. spiralis* seems to be a complex one and may constitute a useful probe into the immune system.

ACKNOWLEDGMENT

This work was supported by the Biomedical Sciences Support Grant RRO 7083.

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