Clin. exp. Immunol. (1977) 29, 496–500.

In vitro cell response of *Treponema pallidum*-infected rabbits

III. IMPAIRMENT IN PRODUCTION OF LYMPHOCYTE MITOGENIC FACTOR

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(*Received* 12 *March* 1977)

SUMMARY

Production of mitogenic factor was examined in rabbits infected intratesticularly with *T. pallidum* and in control animals injected with saline or saline extract of normal rabbits' testes. Lymph nodes and spleen from animals killed 2, 6 and 12 weeks after injection were used as the source of lymphocytes, cultured in serum-free medium in the presence of Reiter antigen. The active supernatants of lymph node cells (LNAS) and spleen cells (SPAS) were examined for the presence of mitogenic factor using normal rabbit peripheral lymphocytes. The LNAS of control animals showed a mitogenic index (MI) between 4 and 6 and the infected animals <2. The SPAS of infected and control rabbits showed an MI of <2. The lower mitogenicity in LNAS of infected and that of SPAS of infected and control animals seems to be due to the presence of inhibitors of DNA synthesis.

INTRODUCTION

The early stage in naturally or experimentally acquired syphilis is characterized by cell immunosuppression (Musher, Schell & Knox, 1974; Pavia, Folds & Baseman, 1976; Wicher & Wicher, 1977a,b). Nevertheless, cells of *T. pallidum*-infected subjects produce *in vivo* or *in vitro* lymphokines upon antigenic stimulation (Fulford & Brostoff, 1972; Schell & Musher, 1974; Wicher and Wicher, 1975), indicating a state of sensitization. As some lymphokines may also be a product of B cells (Petit & Unanue, 1974; Rocklin *et al.*, 1974), the function of T cells during the early stage of infection might still be impaired. Evidence suggests that mitogenic factor is preferentially produced by a T cell-enriched lymphocyte population (Rocklin *et al.*, 1974; Geha & Merler, 1974), and therefore its production has an important bearing on the specificity of the cellular response. This was considered the test of choice to confirm our findings on cell immunosuppression in *T. pallidum*-infected rabbits.

MATERIALS AND METHODS*

Animals. New Zealand rabbits, 3–4 kg each, obtained from the same colony as the animals used for previous experiments were used for this study. A group of eighteen animals was infected with approximately 2×10^7 *T. pallidum* (Nichols strain). Two control groups were included. In the first group ten rabbits were injected intratesticularly (i.t.) with 1 ml of saline extract of normal rabbit testes mixed in equal volume with extract of *T. pallidum*-free infected testes while the second group of ten animals was injected i.t. with 1 ml of saline solution. For convenience, the first control group will be referred to as normal rabbit testes group (NRT) and the second one as saline control group (SC). Animals were handled exactly as described previously. The course of infection and antibody production (as well as lack of such) in the control animals was very similar to that described in the preceding publications.

* The sources and abbreviations of reagents, if not specified, have been reported in the preceding publications.

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Antigen. Reiter antigen (RAg) prepared as described previously (Wicher & Wicher, 1975) was used for these experiments.

Stage I cultures—production of mitogenic factor (MF). Six animals of each group were killed 2, 6 and 12 weeks after injection. Mesenteric and inguinal lymph nodes (LN) and spleens (SP) of infected and control animals were removed aseptically, minced and pressed through a stainless steel sieve into HBSS. The cells were washed twice with HBSS and resuspended to a final concentration of 5×10^6 cells/ml in RPMI 1640 containing 50 u/ml of penicillin and 50 μg/ml of streptomycin. Cell suspensions obtained from spleens were subjected to treatment with ammonium chloride (0·83%) in Tris buffer, pH 7·2, for 2 min at 4°C to lyse erythrocytes. The leucocytes were washed three times with HBSS and resuspended in antibiotic containing RPMI 1640 to give a concentration of 5×10^6 cells/ml.

Cultures were set up as follows: to 10 ml of LN- or SP-cell suspension 10 μg of RAg was added. Control samples received no antigen. No serum was used for the cell culture. The cultures were incubated for 48 hr at 37°C in an atmosphere containing 5% CO_2. At the end of incubation, the control cultures were re-constituted with the same concentration of antigen and all samples immediately centrifuged at 500 *g* for 20 min. The active (AS) and reconstituted supernatants (control) (RS) were filtered through 0·45 μm porous filters, aliquoted and stored at −20°C until used. The active supernatants of lymphocytes obtained from lymph nodes or spleen are abbreviated as LNAS and SPAS, respectively.

Stage II Culture—assay of mitogenic activity. Normal rabbit peripheral blood lymphocytes were separated by the method of Böyum (1968). The purified peripheral lymphocytes (PPL) were washed twice in HBSS and resuspended in antibiotic containing RPMI 1640 with 15% inactivated homologous normal rabbit serum. To triplicate samples, each containing 5×10^5 cells in 0·75 ml volume, 0·25 ml of the undiluted or diluted, active or reconstituted, supernatants of LN or SP cultures were added and incubated for 3 days at 37°C in an atmosphere containing 5% CO_2. 24 hr before harvesting, 1 μCi of [^{3}H]Tdr was added to all cultures. The cells were harvested, processed as described in the preceding publications and the ct/min of triplicate samples averaged. The results were expressed as mitogenic index: (MI) = (AS/RS).

Effect of LNAS on lymphocyte transformation of BCG-immunized rabbits. Three rabbits were immunized with BCG (Lot C265-1, Connaught Laboratory Ltd, Toronto, Canada). 3 weeks later, the peripheral lymphocytes of the PPD-positive rabbits (skin test) were examined. PPL, after washing, were resuspended in RPMI 1640 containing antibiotics and 15% pooled, inactivated, homologous rabbit serum. Triplicate cultures containing 5×10^5 lymphocytes/ml were incubated with 1 μg of PPD alone, or with 1 μg of PPD and 0·25 ml of LNAS of *T. pallidum*-infected (6 weeks), or control (NRT), animals. Triplicate control cultures contained only RPMI 1640. All tubes were incubated for 6 days at 37°C in an atmosphere of 5% CO_2 and processed as previously described. The results are expressed as per cent of response of cultures incubated without LNAS.

Effect of LNAS on allogeneic mixed lymphocyte culture. PPL from three pairs of normal rabbits, after washing, were resuspended in RPMI 1640 containing antibiotics and 15% pooled, inactivated, homologous normal rabbit serum. 0·5 ml of cell suspension containing 10^6 cells/ml from each rabbit was mixed (A × B) and incubated with 0·25 ml of LNAS obtained from *T. pallidum*-infected (6 weeks), or control (NRT), rabbits or with 0·25 ml of RPMI 1640. Controls containing a double number of lymphocytes (1 ml of 10^6 cells) of individual rabbits (A_2 and B_2) were run in parallel. The cultures were incubated for 6 days, and processed as described before. The results were expressed similarly as mentioned above.

All results were analysed by Student's *t*-test.

RESULTS

Examination of LNAS and SPAS for mitogenic activity

Aliquots of AS and RS obtained from cells of LN and SP of infected or control rabbits were pooled and examined at a final dilution of 1:4 in cultures of normal rabbit peripheral lymphocytes. The results (Fig. 1) represent the mean value of six rabbits in each group ± standard error of the mean (s.e.m.). The

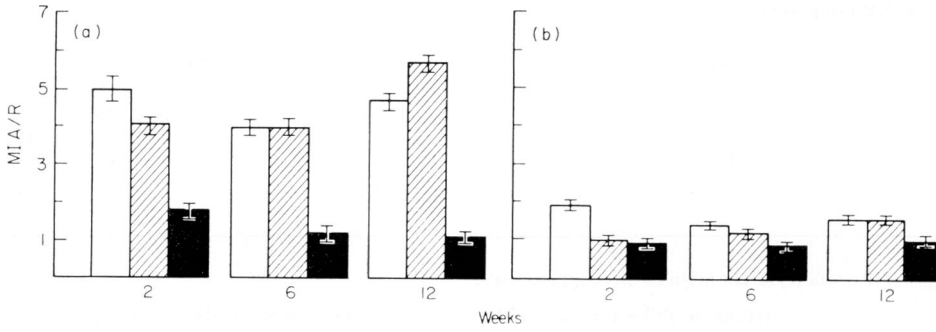

FIG. 1. Determination of mitogenic activity of lymph node (LNAS) and spleen cell (SPAS) active supernatants. The results are expressed as mean values ± s.e.m. MI: mitogenic index; A/R: active supernatant/reconstituted (control) supernatant; (a) lymph nodes; (b) spleen; (□) normal; (▨) NR testis; (■) *T. pallidum*-infected.

supernatant from LN cells of both control groups demonstrated MI between 4–6 while those from the *T. pallidum*-infected rabbits were never higher than 1·8 ($P<0·02$). There was little variation in the results obtained with 2-week- and 12-week-infected, or control, animals. The supernatants of SP cells of all three groups showed comparatively lower mitogenic activity and no significant difference between the infected and control animals was observed. To evaluate the animals' individual variations in mitogenic activity, LN and SP supernatants from infected and control animals were examined, after 6 weeks from the injection. For the quantification of blastogenic activity, the AS and RS were tested at final dilutions of 1:4–1:36. This experiment was repeated four times using lymphocytes from different normal rabbits. The results (Fig. 2) express the mean MI±s.e.m. of six supernatants in each group for each

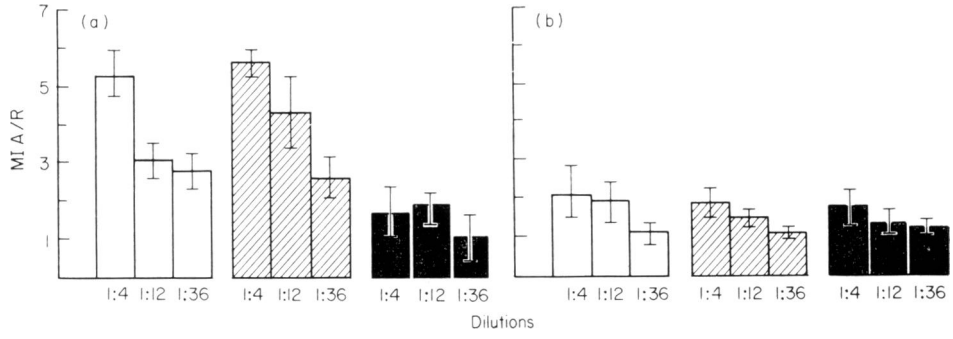

FIG. 2. Quantification of mitogenic activity in LNAS and SPAS of rabbits after 6 weeks of infection. The supernatants were used in dilutions from 1:4 to 1:36. Peripheral lymphocytes of four normal rabbits were used. The results are expressed as group mean value± s.e.m. For key to symbols and columns, see Fig. 1.

TABLE 1. The effect of LNAS on antigen-stimulated lymphocytes

	Antigen-stimulated lymphocytes		Stimulation (%)*	
Source of LNAS	System I	System II	I†	II‡
T. pallidum-infected rabbit no.				
1	BCG-immunized rabbit lymphocytes stimulated with 1 μg PPD alone or PPD with LNAS	Allogeneic mixed lymphocyte cultures, lymphocytes of donors A and B cultured with or without LNAS	48	66
2			43	77
3			111	132
4			31	86
5			76	53
6			60	42
Control (NRT-injected) rabbit no.				
1	As above	As above	278	141
2			113	120
3			260	310
4			159	193
5			171	152
6			200	189

* Results represent mean of three experiments.

† System I: $\frac{(\text{ct/min of PPD-stimulated cells}+\text{LNAS})-\text{ct/min of nonstimulated cells}}{(\text{ct/min of PPD-stimulated cells}+\text{RPMI})-\text{ct/min of nonstimulated cells}}\times 100.$

‡ System II: $\frac{(\text{ct/min of cells A}\times\text{B}+\text{LNAS})-(\text{ct/min of cells }[A_2+B_2/2])}{(\text{ct/min of cells A}\times\text{B}+\text{RPMI})-(\text{ct/min of cells }[A_2+B_2/2])}\times 100.$

dilution examined. LNAS from both control groups produced good stimulation of DNA synthesis in cultures of allogeneic lymphocytes. Significantly lower ($P<0{\cdot}02$) mitogenic activity was observed in cultures incubated with LNAS of *T. pallidum*-infected animals. The dilution affected the activity of the supernatants of control animals but not of infected rabbits. Spleen supernatant fluids, regardless of whether from infected or control animals, showed a uniformly lower degree of mitogenic activity hardly exceeding an MI of 2, and this decreased further with the dilutions.

The effect of LNAS on antigen-stimulated lymphocytes

The question of whether the lower mitogenic activity observed in the AS of infected animals is due to a defect in mitogenic factor production or to the presence of inhibitors of DNA synthesis was answered by the results obtained using PPD-stimulated lymphocytes, from BCG-immunized rabbits, and allogeneic mixed lymphocyte cultures from normal rabbits. The results of both experiments are shown in Table 1. Under the experimental conditions applied, five out of six LNAS from the *T. pallidum*-infected group showed a definite decrease in DNA synthesis (31–76% stimulation in PPD-stimulated culture, 42–86% in allogeneically stimulated cultures). The supernatant of rabbit No. 3 repeatedly failed to inhibit the lymphocyte cultures. An increased stimulation (113–278% in PPD-stimulated cultures, 120–310% in allogeneically stimulated cultures) was observed with all LNAS from the control animals. The difference in the responses of both groups is significant ($P<0{\cdot}01$).

DISCUSSION

Since the rôle of T cells in *T. pallidum*-infected subjects is not known with certainty, we have chosen to examine the production of mitogenic factor in infected rabbits, because it is considered to be associated primarily with T-cell function (Geha & Merler, 1974; Rocklin *et al.*, 1974) and, therefore, to be a more reliable indicator of cell-mediated immunity. Our studies demonstrated that LN, but not SP, lymphocytes of normal rabbits, after interaction with RAg, produce mitogenic factor. Production of such a factor was impaired in rabbits infected i.t. with *T. pallidum*. The response of lymphocytes from normal subjects to *T. refringens* (Musher *et al.*, 1974) and to *T. pallidum* (Friedman & Turk, 1975) has been reported. The response of lymphocytes from normal rabbits or man to various treponemal antigens could most likely be explained by cross-sensitization of lymphocytes by endogenous treponemes colonizing animals or man. The impairment of mitogenic factor production in the infected animals might have a more complicated underlying mechanism, and it is more difficult to explain.

The high mitogenic activity observed in the present experiments with lymphocytes from normal rabbits stimulated by RAg contrasted with that observed in our previous study (Wicher & Wicher, 1977a), using the direct lymphocyte transformation test. The larger total number of cells/ml used in the two-step method could probably explain the difference in the quantity of released factor. However, the factual reason for the difference in the mitogenic activity is unknown. While the release of blastogenic substances might effectively be accomplished, the recruitment and/or amplification could somehow be impaired in direct lymphocyte transformation. Whether this is a property inherent in rabbit lymphocytes, or whether it depends on the characteristics of the antigen used remains to be established.

The suppressed production of mitogenic factor in the two-step method must be attributed to the infection, since LNAS of control animals demonstrated mitogenic activity exceeding two to three times that of the infected animals. These results, in general, confirm our previous observations (Wicher & Wicher, 1977a) and those of Pavia *et al.* (1976) concerning the reduced response to mitogens of lymphocytes in *T. pallidum*-infected rabbits. SPAS from all animals showed a lower level of mitogenic activity (MI $\leqslant$ 2) than the LNAS. No significant differences were observed among infected and control animals. The different biological behaviour of LN and SP lymphocytes remains to be examined. However, we may speculate that, when introducing the treponemes i.t., the organ primarily affected, in addition to the testes, is the lymph node. Furthermore, it is known that different sub-populations of lymphocytes occur in different lymphoid organs and, therefore, various cellular interactions and co-operations might be expected (Gershon, 1975).

The question of whether, in the infected animals LNAS, there is a lack of, or decreased, blastogenic activity, or increased production of inhibitory substances, was explored using lymphocyte transformation of PPD-sensitive cells and allogeneic mixed lymphocyte cultures. The results indicated the presence of DNA-synthesis inhibitor(s) in the LNAS from infected rabbits. The presence of suppression and/or enhancement of lymphocyte activity is consistent with the heterogenicity of factors produced *in vitro* by antigenically stimulated lymphocytes (Kasakura, 1970) and its expression will depend upon the test system used to detect it. The inhibitor(s) is(are) most probably of cellular origin since supernatants were produced in serum-free medium. The cells involved in the production of inhibitors are unknown. Suppressor activity has been found in a sub-population of T lymphocytes (Taniguchi & Tada, 1974), although the factor inhibitory to proliferative response in the mouse has been suggested to be a product of macrophages (Nelson, 1972) or B cells (Veight & Michael, 1972). The level of mitogenic activity found in control SPAS (MI = 1·10–2·0) and infected animals (MI = 1·19–1·70) was similar to that obtained with LNAS from infected animals. In addition, the effect of both SPAS (infected and control) on PPD and allogeneically stimulated cultures (results not reported here) was similar to that of infected LNAS, i.e. decreased DNA synthesis. The relationship between this inhibitory activity and that found in the serum of syphilitic rabbits (Wicher & Wicher, 1977b) is highly suggestive of some correlation and is presently under investigation. In pursuing this, we are probably close to understanding the complexity of the problem and, most of all, the discrepancies observed during the early stages of syphilitic infection.

We thank Rita Belani for her excellent technical assistance. This work was supported by grant R01-AI-11102 from the National Institute of Health, United States Public Health Service.

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