Inability of Spleen Cells from Chancre-Immune Rabbits to Confer Immunity to Challenge with *Treponema pallidum*

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Received for publication 11 April 1977

Although several lines of evidence suggest that cellular immune mechanisms play a role in controlling infection due to *Treponema pallidum*, recent studies have shown that induction of acquired cellular resistance by antigenically unrelated organisms fails to protect rabbits against syphilitic infection, thereby casting doubt on this hypothesis. In the present paper we describe attempts to transfer immunity to syphilis by using spleen cells from chancre-immune rabbits. Intravenous infusion of 2×10^8 spleen lymphocytes was capable of transferring acquired cellular resistance to *Listeria* and delayed hypersensitivity to tuberculin. However, in eight separate experiments using outbred or inbred rabbits, 2×10^8 spleen cells from syphilis-immune animals failed to confer resistance to *T. pallidum* whether by intravenous or intradermal challenge. Mixing immune lymphocytes with treponemes immediately before intradermal inoculation also failed to confer resistance. Despite the fact that syphilitic infection stimulates cellular immune mechanisms and induces acquired cellular resistance to antigenically unrelated organisms, cellular immunity may not play an important role in immunity to syphilis.

A number of observations have suggested that cell-mediated immunity (CMI) plays an important role in the response of the host to syphilitic infection. Delayed hypersensitivity to *Treponema pallidum* appears late in secondary syphilis, just before the onset of latency, and is uniformly present in late latent and tertiary syphilis (23, 36). Previous studies from our laboratory showed that *T. pallidum* infection in rabbits induces acquired cellular resistance (ACR) as manifested by the ability of the host to suppress the growth of *Listeria monocytogenes* (30). This resistance could be transferred to other rabbits by using thymus-dependent spleen cells and treponemal antigen (31, 32). In contrast, nonspecific macrophage activation due to infection with *Mycobacterium bovis* (strain BCG) and *Propionibacterium acnes* did not alter the course of infection due to *T. pallidum* (4, 14, 33). Even though heterologous stimulation of ACR is not expected to be as effective as homologous reactivation (10, 14), the absence of any protection argues against an important role for CMI. The most direct way of demonstrating a protective role for CMI mechanisms would be to confer immunity to *T. pallidum* infection on virgin recipients by transferring lymphocytes from syphilis-immune animals. The results of

such experiments are described in the present paper.

MATERIALS AND METHODS

Animals. Outbred New Zealand white male rabbits and inbred strain III VO/J rabbits (Jackson Laboratories, Bar Harbor, Me.) weighing 2 to 3 kg were housed individually in stainless-steel cages at an ambient temperature of 18°C, a condition which facilitates the development of disseminated syphilitic lesions (39). Rabbits with a reactive VDRL or FTA-ABS test were excluded because of the possibility that this resulted from a subclinical infection with *Treponema cuniculi*. In all phases of this study, rabbits were randomly selected as donors of spleen cells or recipients for challenge with *T. pallidum*.

***T. pallidum*.** The Nichols strain of *T. pallidum* was maintained by intratesticular passage. Infected testes were removed aseptically from rabbits that had been treated with cortisone acetate (38); the testes were minced in sterile minimal essential medium (MEM) and ground with sand, using a mortar and pestle. After centrifugation at $754 \times g$ for 10 min to remove cellular debris, the number of treponemes in the supernatant was determined by dark-field microscopy. Suspensions of *T. pallidum* for inoculation of rabbits were obtained from serial dilutions in MEM. In studies using inbred rabbits, recipient animals were always challenged with treponemes obtained from the inflamed testes of another inbred rabbit.

Preparation of spleen cells for adoptive cell transfer. Single-cell suspensions of normal or syphilis-immune splenic lymphocytes were obtained by pressing minced spleens through 80 $\times$ -mesh, sterile, stainless-steel screens into petri dishes containing MEM and 10 U of heparin per ml. Cell clumps and debris were removed by sedimentation on a 10-cm column for 10 min. The material that had settled was discarded, and the supernatant containing single cells was centrifuged at 600 $\times$ *g* for 10 min. The cell pellets were exposed briefly to 0.84% ammonium chloride to remove contaminating erythrocytes. MEM was added to the treated suspensions, and the cells were washed twice to eliminate toxic subcellular debris and resuspended for counting. This procedure did not cause loss of viability as determined by the ability of cells to exclude 0.1% trypan blue. Furthermore, ammonium chloride treatment, as performed in our laboratory, has not been shown to adversely affect the ability of splenic lymphocytes to respond to mitogens in vitro or to respond in plaque-forming assays (R. E. Baughn and D. M. Musher, unpublished observations).

The status of immune donors and the number of lymphocytes transferred to recipient animals are explained in the text for each experiment. Preliminary adoptive transfer studies suggested that recipients receiving infusions of more than 5×10^8 cells in 10 ml of medium experienced labored breathing. To preclude this from happening in the inbred animals, recipients were pretreated with 500 U of heparin intravenously.

Challenge of recipient animals with *T. pallidum*. In most experiments, rabbits were challenged either intradermally (i.d.) with 10^3 or 10^4 *T. pallidum* or i.v. with 4×10^7 organisms. All inoculations were completed within 45 min after the rabbit with the syphilitic orchitis that was serving as a source of treponemes had been sacrificed and within 2 h of the adoptive transfer of lymphocytes. In addition to dark-field quantitation of organisms, in each experiment one normal rabbit was injected with graded doses of *T. pallidum* to verify the treponemal counts.

Recipient rabbits were shaved 2 to 3 days before the experiments to exclude "rapid hair growers"; animals that were not rapid hair growers served as recipients and were shaved 3 to 4 times weekly throughout the course of observations. The course of syphilitic infection was observed by daily examination of each rabbit. Induration, erythema, and necrosis of dermal lesions were evaluated by using a recently described modification (41) of the method of Turner et al. (38).

Mixed-lymphocyte reactions on inbred splenic lymphocytes. Mixed-lymphocyte reactions were performed to verify the genetic stability of the III VO/J-strain rabbits. Spleen cells from individual rabbits were treated with mitomycin C by the method of Bach and Voynow (3), and one-way responses were assessed according to standard methods (40).

RESULTS

Preliminary studies in outbred New Zealand white rabbits. A series of adoptive transfer studies was initially conducted with outbred animals as donors and recipients. Although decreased survival of lymphocytes was expected in

these animals by virtue of the fact that skin grafts are normally rejected after 10 to 12 days, we hoped to show that early interactions between immune lymphocytes and treponemes within the first few days of transfer might modify the course of infection. Previous studies from our laboratory had used outbred rabbits to show that ACR (31, 32) and delayed hypersensitivity could both be transferred via splenic lymphocytes.

In the first experiment, four rabbits received 10^8 normal splenic lymphocytes (NSL) and four received splenic lymphocytes from syphilis-immune rabbits (ISL); four control animals received no spleen cells. The immune donors in this experiment were rabbits that had been infected i.v. with *T. pallidum* 2 months before sacrifice; lesions in these animals had completely resolved. Two rabbits in each group were then challenged i.d. with 10^3 *T. pallidum* at six different sites, and two were challenged i.v. with 4×10^7 organisms. Dermal chancres appeared 11 to 12 days later at all sites in those animals infected i.d., and disseminated lesions appeared 17 to 21 days later in rabbits infected i.v. There were no apparent differences in the size or extent, or in the rapidity of resolution, of lesions in any of the three groups. This experiment was repeated later under identical conditions except that recipients received 10^9 NSL and ISL. Again no differences were noted among the various experimental groups.

Because of the possibility that decreased survival of lymphocytes might have been responsible for failure to confer immunity in the above experiments, recipient rabbits in the third experiment received 10^9 ISL 7 or 14 days after i.v. challenge with *T. pallidum*. In addition, two rabbits received ISL on the day of challenge and two rabbits were given no spleen cells. All rabbits developed disseminated syphilitic lesions within 15 to 18 days of challenge, with no differences being detected among the different groups.

Adoptive transfer studies in III VO/J rabbits. Because of the possibility that lymphocyte rejection had been responsible for the failure to transfer immunity in outbred animals with ISL, additional experiments were conducted with strain III VO/J inbred rabbits. These rabbits have an inbreeding coefficient (*F*) of 0.96 and are able to maintain skin grafts for more than 19 months. In a preliminary attempt to verify the genetic stability of the inbred strain and to assess the survival of splenic lymphocytes in recipients, one rabbit was vaccinated i.v. with 3×10^7 BCG-Tice [lot IL 74 (s) 11, obtained from the Research Foundation, University of Illinois, Chicago]. Three weeks

later this animal was skin test positive when tested with 10 μ g of purified protein derivative (PPD; Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y.). This animal then received a booster of 10^7 BCG-Tice 1 week later and was sacrificed 3 weeks later. Splenic lymphocytes (2×10^8) were transferred i.v. to each of three PPD-negative inbred rabbits. These recipients were skin tested 14 days after the adoptive transfer of cells and exhibited skin reactions ranging in size from 9 to 15 mm and 11 to 18 mm of erythema at 24 and 48 h, respectively, at the injection sites of 10 μ g of PPD. In addition, one-way mixed-lymphocyte reactions were performed with spleen cells from donor rabbits to verify the genetic stability of the strain. The results of one study on four donor rabbits are shown in Table 1. The background or nonspecific activity of donor lymphocytes was subtracted in computation of the data. Cells from each donor were capable of responding to phytohemagglutinin (PHA), but were not stimulated by mitomycin C-treated cells from the donors. One-way mixed-lymphocyte reactions at the end of 3 to 6 days in culture also failed to detect histocompatibility differences, although the responses to PHA at these times were less than at 48 h (Table 1).

TABLE 1. One-way mixed-lymphocyte reactions of spleen cells from inbred donors

Responder cells	Addition	Log ₁₀ cpm/ 10^6 cells ^a
50471 ^b	52099-M ^c	-0.28
	52193-M	0.25
	55050-M	-0.19
	50471-M	-0.04
	PHA	1.10
52099	50471-M	0.32
	52193-M	0.22
	55050-M	-0.09
	52099-M	-0.04
	PHA	1.28
52193	50471-M	0.09
	52099-M	0.23
	55050-M	0.00
	52193-M	-0.27
	PHA	1.26
55050	50471-M	0.13
	52099-M	0.18
	52193-M	0.36
	55050-M	0.27
	PHA	1.72

^a Cell cultures were pulsed with 1 μ Ci of [³H]thymidine for the last 24 h of a 48-h incubation period. Log₁₀ of background (donor + donor-M) was subtracted.

^b Jackson Laboratories identification number.

^c The designation -M indicates that stimulator cells were treated with mitomycin C.

Initial studies in our laboratory also confirmed that the course of syphilis in these rabbits was similar to that in outbred animals, although there was some increased variability in the time that lesions appeared after treponemal challenge.

In the first adoptive transfer study, donors were infected i.v. with 4×10^7 *T. pallidum* 31 days before the time of sacrifice. Previous studies have shown this to be a time at which ACR to *Listeria* is maximally enhanced. At this time, disseminated lesions that had appeared in these animals were regressing. Two recipient rabbits per group received 2×10^8 NSL, 2×10^8 ISL, or no spleen cells. One hour later, all six animals were challenged with 4×10^7 *T. pallidum* in the contralateral ear vein. No differences were detected in the time of appearance of lesions, their number, or the degree of severity. In a replicate of this experiment, two animals in each group received 5×10^8 NSL or no spleen cells, and four were given 5×10^8 ISL from donors that had been infected i.v. 3 months earlier with *T. pallidum*. This time interval was chosen because of Turner's hypothesis that immunity to syphilis in rabbits reaches its peak at this time (37). All the animals were then challenged i.v. with 4×10^7 *T. pallidum* (Table 2). Once again no protection could be observed in rabbits that had received lymphocytes from syphilis-immune donors.

In the final two experiments, inbred recipients were challenged i.d. with 10^3 *T. pallidum* at each of six sites. Immune lymphocytes were administered i.v. to some recipients immediately before i.d. challenge. Because of the possibility that systemically administered lymphocytes do not reach dermal sites of injection, other rabbits were injected i.d. with an admixture of ISL and treponemes at a lymphocyte-treponeme ratio of 1,000:1. Control animals received 10^3 *T. pallidum* admixed with NSL in the same proportion or 10^3 *T. pallidum* alone. Rabbits that received ISL i.v. followed by *T. pallidum* i.d. developed chancres that were indistinguishable from those in recipients of *T. pallidum* admixed with NSL or *T. pallidum* alone (Fig. 1). Rabbits that received ISL admixed with *T. pallidum* had early dermal reactions at the sites of injection manifested by ≤ 5 -mm erythema without induration. These subsided within 7 to 8 days, and typical syphilitic chancres subsequently developed at the same rate and with the same progression pattern as in the other three groups.

The appearance of VDRL and FTA-ABS antibodies was also evaluated by standard techniques. Serum was obtained 5, 10, and 15 days after challenge with *T. pallidum* and weekly

thereafter. FTA-ABS reactions were routinely 4+ on 1:100 dilutions of serum obtained early (days 5 and 10) from those recipients infused with ILS. FTA-ABS antibody reactions of a similar magnitude were not seen in the control animals until day 21. VDRL reactivity could not be correlated with either the transfer of lymphocytes or the appearance of generalized lesions in any of the groups.

DISCUSSION

The nature of immunity in syphilis remains poorly understood (23, 37). Early studies concentrated on humoral responses, showing that specific complement-dependent antibodies that inhibit motility and infectivity of *T. pallidum* are present in the serum of patients with secondary syphilis (39). These antibodies do not prevent the progression of syphilis to secondary and tertiary stages. Moreover, passive humoral immunization delays, but does not eliminate, the appearance of syphilitic lesions after i.d. challenge.

Favoring a role for CMI is the observation that delayed hypersensitivity to *T. pallidum* appears late in secondary infection and is regularly present in latency (6, 13, 23, 24). Moreover, tertiary syphilis is characterized by the formation of granulomas indistinguishable from those that are seen in other granulomatous conditions (37).

In vitro studies of the blastogenic response of lymphocytes during syphilis have provided further indirect evidence for a role of CMI. Suppressed response to PHA (11, 17, 20, 25), concanavalin A (28, 29), treponemal antigens, including *T. refringens*, *T. reuteri*, and *T. pallidum* (24, 25), and nontreponemal antigens such as *Candida* and trichophytins (24, 25) has been demonstrated in syphilis. After treatment for syphilis, lymphocyte responsiveness returns to normal (25). M. Metzger has shown that lymphocytes from syphilitic rabbits produce substances in vitro that inhibit the migration of leukocytes (personal communication). In contrast,

FIG. 1. Inability of ISL to influence the onset and progression of cutaneous lesions in rabbits. The mean lesion scores were obtained from six sites in: four rabbits receiving 10^3 *T. pallidum* alone per site (—); two rabbits receiving 10^3 *T. pallidum* per site after i.v. infusion of 5×10^8 ISL (---); four rabbits receiving 10^3 *T. pallidum* admixed with 10^7 NSL per site (x); and four rabbits injected with 10^3 *T. pallidum* admixed with 10^7 ISL per site (- · - ·).

other investigators have shown that treponemal extracts stimulate leukocyte migration (12, 42) early and suppress migration late in syphilis. These observations are difficult to interpret because of the relatively small differences in migration and the unusual nature of the observed response. Finally, Friedmann and Turk (11) found that patients with syphilis had diminished reactivity to tuberculin but enhanced reactivity to a preparation of *T. pallidum*. All these studies provide indirect evidence that CMI is invoked by syphilitic infection.

Suppression of bacterial growth in the spleens and/or livers of animals after an i.v. challenge with *L. monocytogenes* has been used to assess nonspecific manifestations of CMI responses invoked by antigenically unrelated organisms (21). We have shown that the growth of *Listeria* is suppressed between 3 and 6 weeks after i.v. infection with *T. pallidum* (30). Using sensitized lymphocytes from syphilitic rabbits and *T. pallidum* as the sensitizing antigen (22), it was possible to transfer acquired cellular resistance to *Listeria* (31, 32). Antithymus serum abolished this transfer of resistance. These observations show that *T. pallidum* stimulates ACR. If CMI plays a central role in immunity to infection with *T. pallidum*, macrophage activation should protect rabbits against syphilis. Several studies have failed to demonstrate this. Rabbits

TABLE 2. Development of dermal syphilitic lesions in rabbits receiving immune splenic lymphocytes and challenged i.v. with *T. pallidum*

Experimental group	Identification no.	Source of donor lymphocytes	Incubation period (days)		
			Earliest rash	Disseminated lesions	Orchitis
Control	51175	None	20	27	32
	44188		36	38	
Control	51907	5×10^8 NSL	23	29	31
	42867		25	31	
Test	51202	5×10^8 ISL	23	27	30
	51006		25	29	31
	41355		23	28	33
	41149		27	33	

infected with *M. bovis* (strain BCG) (14, 33) or sensitized with *P. acnes* (4) were not resistant to i.d. or i.v. challenge with *T. pallidum* (14, 33).

A classic feature of infections that are mediated by CMI is the persistence of infecting organisms within macrophages (9, 13, 16, 21). Some investigators have found treponemes intracellular in both rabbit and human tissue after infection with *T. pallidum* (2, 3, 19, 27, 34, 35). However, there is little to suggest that these organisms are persisting in a viable state, much less replicating; in fact, they usually appear to be deteriorating, presumably due to enzymes and/or other substances within vacuoles. Moreover, although they are usually found in macrophages (19, 27, 34, 35), they can be found within lymphocytes, plasma cells, and epithelial cells, and extracellular organisms predominate in all stages of infection.

These results suggest that, although infection with *T. pallidum* induces ACR, this nonspecific resistance may not play a major role in controlling syphilitic infection. Although it has generally been thought that infections which stimulate CMI are brought under control by these same nonspecific mechanisms (21), recent studies (10, 15) have questioned this concept. Syphilis may be another infection that does not follow the more "classic" pattern (10).

The studies described in this paper were designed to provide evidence that CMI plays a functional role in protection against *T. pallidum* infection. Using numbers of spleen cells that are known to transfer resistance in mice (22) and rabbits (31, 32), we attempted to transfer chance immunity from rabbits that had recovered from syphilis and were refractory to rechallenge. After i.v. or i.d. injection of *T. pallidum*, recipients of immune spleen cells developed syphilitic infections that were indistinguishable from those in controls. It was possible that initial studies using outbred rabbits failed because of lymphocyte rejection, although the various times of cell transfer, the inocula of *T. pallidum*, and the expected incubation periods might have been expected to bypass this problem. Accordingly, these experiments were modified and repeated with III VO/J inbred rabbits. Genetic stability was verified by mixed-leukocyte reactions, and transferability of cell-mediated responses was verified by using splenic lymphocytes to transfer tuberculin reactivity. Despite the presence of high-grade chance immunity in donor rabbits, demonstrated by their failure to develop chancres after i.d. challenge with 10^7 *T. pallidum*, there was no protective effect of immune lymphocytes in recipients.

The events that occur in vivo with respect to the interaction of immune lymphocytes with

antigen, culminating in macrophage activation, are not known (7). It is possible that sufficient numbers of systemically administered immune lymphocytes do not "home" or are not recruited to sites of antigen deposition. Thus, in turn, trapping of monocytes (1) and subsequent activation of macrophages would not ensue. Although such a possibility seemed unlikely based on the ability to transfer tuberculin reactivity, two final sets of experiments included rabbits that received immune spleen cells i.d. together with *T. pallidum*. Although these animals demonstrated a local reaction at 3 to 6 days that was consistent with delayed hypersensitivity, they still developed chancres that progressed at the same rate and eventually grew to the same size as controls. It is interesting to note that recipients of immune cells developed FTA-ABS more rapidly than controls, suggesting that memory B-cells were transferred.

One could argue that one of the reasons for the failure to transfer immunity was the choice of splenic lymphocytes rather than lymph node cells. Although we did not demonstrate the presence of immune T-cells specifically directed against treponemal antigens in our spleen preparations, splenic lymphocytes have generally been used for such studies (21, 22) and have specifically been shown to be more efficient than lymph node lymphocytes in some adoptive transfer experiments (43). Moreover, the search for lymph nodes (other than mesenteric) is relatively unrewarding in normal rabbits, and it would not have been feasible to carry out appropriate controls had we attempted to use lymph node cells. It is of further interest that rabbits are unable to effect a biological cure without the aid of antibiotics and continue to harbor virulent *T. pallidum* in their lymph nodes for life (39). Overall, the results of these studies, together with those (4, 14, 33) previously cited, suggest that CMI mechanisms do not play a central role in chancre immunity.

ACKNOWLEDGMENTS

We wish to thank Donald Winn for his excellent technical assistance and Mona Thomas for secretarial assistance.

This work was supported by Public Health Service grant AI 12618-02/05 P-1 from the National Institute of Allergy and Infectious Diseases and by research funds from the Veterans Administration Hospital, Houston, Tex.

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