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Antibody-mediated immunity in CFW mice infected with *Mycobacterium lepraemurium*

HUMORAL IMMUNE RESPONSE IN MURINE LEPROSY

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SUMMARY

A depression in antibody-mediated immunity (AMI) measured both in terms of circulating antibody and plaque-forming cells in the spleen was observed in CFW mice infected with *M. lepraemurium* when sheep red blood cells (SRBC) and human gammaglobulin (HGG) were used as antigens. The impairment in AMI was evident only after 75 days of infection thereafter the antibody response to SRBC antigen progressively decreased until the last day of experimentation (135 days). Within the first 60 days of infection no alteration in AMI was observed with the HGG antigen while the response to the SRBC antigen was significantly higher in the infected animals than in uninfected controls.

INTRODUCTION

Since the discovery of rat leprosy as a chronic granulomatous disease caused by an acid-fast bacillus (Dean, 1903; Stefansky, 1903), striking similarities with human lepromatous leprosy have been recognized. Both the aetiological agents, *Mycobacterium lepraemurium* and *M. leprae* and the diseases they cause share several characteristics. Neither organism has been successfully grown in conventional bacteriological media, and they are antigenically (Estrada-Parra, Rojas-Espinosa & Reyes-Gomez, 1968; Reyes-Gomez *et al.*, 1968; Kwapinski & Kwapinski, 1973) and structurally (Sato & Imi, 1968) related. Several reports have appeared which indicate a depression in cell-mediated immunity (CMI) in both human lepromatous and murine leprosy, but no depression in humoral immunity has been found. In this paper we present evidence that the humoral immune response (HIR) is depressed in mice with long-lasting leprosy.

MATERIALS AND METHODS

M. lepraemurium. The Hawaiian strain of *M. lepraemurium* obtained originally from Dr Y. T. Chang (National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland) and maintained in our laboratory by serial infection of CFW mice was used.

Infection of mice. Lots of 100–150 white CFW mice of either sex and 1·5 or 2·5 months of age were inoculated i.p. with about 22×10^7 bacilli per mouse, and fed purina laboratory chow (Ralston Purina Co., St. Louis, Missouri) *ad libitum* thereafter. The inoculum was prepared from s.c. lepromata removed from mice 4 months after infection, trimmed of associated fat and homogenized in a glass Potter–Elvehjem homogenizer in saline. Gross cell debris and bacillary clumps were eliminated by low speed centrifugation and bacilli in the supernatant were washed twice in sterile saline, resuspended in saline, stained and counted.

Assay for plaque forming cells. At intervals after infection, 4–8 mice were inoculated i.p. with 0·15 ml of a 20% suspension of washed sheep red blood cells (SRBC) and 4 days later they were killed by exsanguination via the periorbital venous sinus. The sera were separated and kept frozen until serological determinations were carried out. Immediately after death, spleens

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were removed, weighed and used to assay the number of PFC according to the Jerne technique (Jerne & Nordin, 1963) with minor modifications i.e. appropriate aliquots of the spleen cell suspension in TC Hanks's solution (Difco, Detroit, Michigan) and 0·15 ml of a 10% SRBC suspension, were admixed with 2 ml of melted (45°C) agarose made up 0·7% in TC MEM solution (Difco) and poured into small (60 × 15 mm) plastic Petri dishes (Falcon Plastics, Oxnard, California). After gelification, the plates were incubated for 90 min at 37°C, under a 5% CO_2–95% air atmosphere, before the addition of 1·5 ml of fresh SRBC-absorbed guinea-pig serum diluted 1:30 with Hanks's solution. Readings were taken after an additional 40-min incubation at 37°C. Only 19S antibody producing cells (direct plaques) were assayed.

Immunization with human gamma globulin (HGG). Mice were inoculated subcutaneously with 1 mg of HGG in incomplete Freund's adjuvant (Campbell *et al.*, 1970) and 100 μg of heat-aggregated (63°C, 20 min) HGG in saline 1 week later. Five days later they were bled.

Antibody titrations. Titrations were done on 0·025-ml samples with the aid of a microtitration kit (Microtiter, Cooke Engineering Co., Alexandria, Virginia). Serial two-fold dilutions of serum made in triethanolamine buffer solution (TBS) containing 0·05% gelatin (Difco), were mixed with one volume (0·025 ml) of a 1·5% suspension of SRBC or of tanned SRBC coated with HGG (Campbell *et al.*, 1970) and incubated for 30 min at 37°C. Tests were read immediately and again after overnight incubation at 4°C. All determinations were set up in duplicate. Titres are given as the reciprocal of the highest dilution to give clear agglutination; doubtful results were considered negative.

Antibody to mycobacterial antigens. Serum antibodies to mycobacterial antigens were detected by the double diffusion technique (Ouchterlony, 1949). A cell-free mycobacterial extract containing 0·63 mg of protein per ml and prepared from *M. lepraemurium* as indicated elsewhere (Rojas-Espinosa *et al.*, 1972) was used as antigen.

Protein determinations. Total serum protein was determined by the Biuret method. Electrophoretic fractionation and analysis was done in a Gelman clinical electrophoresis system (Gelman Instrument Co., Ann Arbor, Michigan). Microdeterminations of protein were done by the Lowry method. (Lowry *et al.*, 1951).

RESULTS

Changes in serum proteins

Normal mouse serum can be separated into six or seven main components on the basis of electrophoretic mobility on cellulose acetate which probably correspond to the albumin, α1-, α2-, β1-, β2-, and gammaglobulin fractions of human serum. Patients with lepromatous leprosy display a consistent hypergammaglobulinaemia with occasional inversion of the albumin/globulin ratio (A/G ratio).

Electrophoretic fractionation of serum from normal and infected mice revealed a progressive hypergammaglobulinaemia that became more evident as the disease advanced (Fig. 1). α2-globulins were not consistently elevated. The albumin/gammaglobulin (A/GG) ratio of normal mice was higher than 3·0 (4·36 ± 0·26) during the 135 days of the experiment but infected mice began to show a decrease in the A/GG ratio after 60 days which progressed from normal values on day 0 to about 1·7 on day 135. Later determinations were not done as the mortality in the population was very high after 5 months of infection.

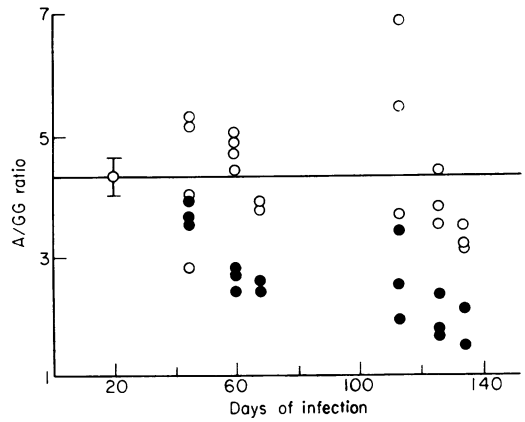

FIG. 1. Albumin/gammaglobulin (A/GG) ratios plotted against time of infection with *M. lepraemurium*. A/GG ratios for both, normal (open circles) and infected (closed circles) mice, were calculated from electropherograms of sera obtained from animals injected i.p. with 0·15 ml of a 20% suspension of SRBC 4 days before they were killed. The vertical bars represent ± one s.e. of the mean.

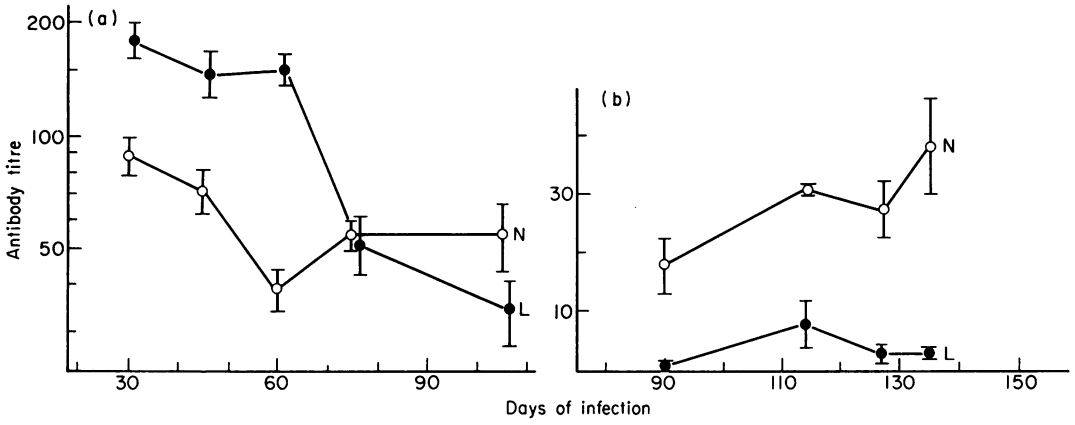

FIG. 2. Antibody production to SRBC-antigen in mice related to the time of infection with *M. lepraemurium*. (a) within the first 105 days of infection. (b) beyond 90 days of infection. Group (a) was 1 month older than group (b) at the time of infection. Normal mice (N) were of the same age and sex as infected animals (L) in groups (a) or (b). The vertical bars are ± one s.e. of the mean.

Antibody responses

During the first 75 days after infection animals given HGG responded like controls. Within the first 60 days of infection, the serum antibody response to SRBC antigen was even higher in infected mice than in controls (Fig 2). On the 90th day after infection, the antibody response to both antigens was markedly depressed in the infected group. The titre of antibodies against SRBC remained low to the last day of the experiment (135 days). The antibody response to HGG was not followed beyond 90 days. (Animals in the experiment illustrated by Fig. 2a, were 1 month older at the time of infection than those used in the experiment illustrated by Fig. 2b).

Plaque-forming cells

As we did not find any consistent difference between infected and normal animals during the first 60 days of infection, these data are not included. After this period infected animals showed a decrease in their ability to produce PFC in parallel to their inability to produce normal amounts of antibody against SRBC. This decrease was still evident after 110 days of infection, and continued beyond this time.

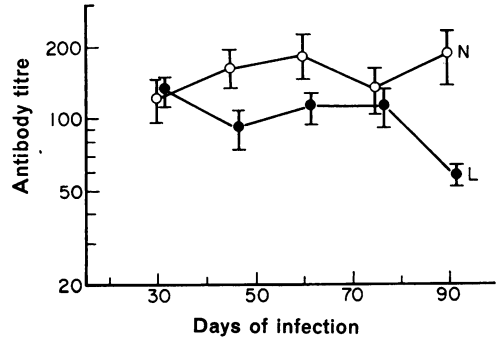

FIG. 3. Antibody production to HGG antigen in mice related to the time of infection with *M. lepraemurium*. Infected (L) and normal (N) animals, from the same group as in Fig. 2a, were 2·5 months at the time of infection. Infected and normal mice received two doses (1 mg and 0·1 mg) of HGG. The vertical bars are ± one s.e. of the mean.

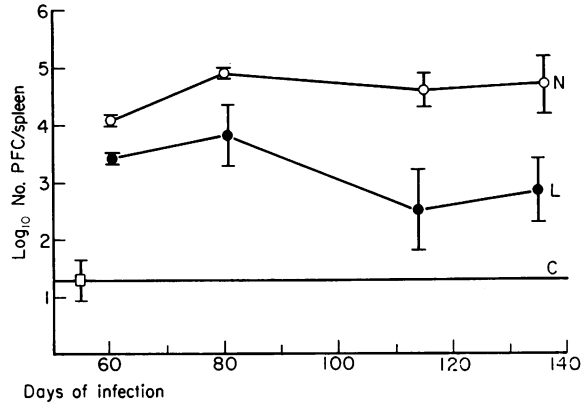

FIG 4. PFC response to SRBC-antigen in mice related to time of infection with *M. lepraemurium*. Normal (N) and infected (L) mice received SRBC (0·15 ml of a 20% suspension i.p.) 4 days before the assay. Two normal unimmunized animals (controls, C) were included at each time. The vertical bars represent ±1 s.e.m.

Although only three normal and three infected animals were studied at each time, the group of infected mice taken as a whole (twelve animals, from 60 to 135 days of infection), showed a low PFC response which was statistically different from the response obtained with the group of normal controls (P = 0·001, Student's t-test for small number of samples). After 110 days, some of the infected animals showed a response which was within the range produced by normal un-immunized animals. The indirect PFC number was not studied because no discrepancy between direct PFC number and haemagglutinin titres was observed (both determinations were done for the same animals).

TABLE 1. Mean antibody titres to SRBC and HGG antigens found in the serum of normal and *Mycobacterium lepraemurium*-infected CFW mice

	Normal			Infected		
Days*	No. of animals†	Mean ± s.e.‡		No. of animals	Mean ± s.e.	
			SRBC-Antigen			
30	4 (8)	88·0	11·7	5 (10)	179·2	20·9
45	6 (13)	71·4	9·7	6 (12)	146·6	21·0
60	5 (9)	39·1	4·7	6 (12)	149·3	14·4
75	5 (10)	54·4	5·3	6 (12)	50·6	9·0
105	5 (11)	53·9	10·8	4 (8)	34·0	7·0
90	4 (8)	18·0	4·7	4 (8)	0·9	0·5
114	6 (11)	30·5	1·5	6 (12)	8·2	3·8
127	6 (12)	27·3	5·2	6 (12)	3·0	1·4
135	6 (12)	38·2	7·4	4 (8)	3·0	1·1
			HGG-Antigen			
30	7 (14)	120·8	25·0	7 (14)	132·3	15·6
45	6 (12)	158·8	28·4	6 (12)	90·0	15·8
60	7 (14)	180·8	39·5	7 (14)	110·0	17·0
75	8 (16)	132·0	28·9	8 (16)	113·3	18·2
90	5 (10)	183·2	46·9	5 (10)	57·2	4·3

* Days after infection with 22×10^7 bacilli per mouse. For the 30–105-day period post-infection, mice of 2·5 months old at the time of infection were used, while for the 90–135-day period, mice having 1·5 months of age at the moment of infection were used.

† The number of animals studied and the total number of determinations are shown in parentheses.

‡ Titres are given as the mean value ± one s.e.m.

Antibody to mycobacterial antigens

By using the Ouchterlony double diffusion method, we were unable to show any evidence for the presence of anti-mycobacterial antibody in the sera of the infected mice throughout the course of the experiment. By contrast, three out of fourteen serum samples obtained from patients with diffuse lepromatous leprosy (DLL), gave precipitin bands with the same mycobacterial preparation (unpublished work).

DISCUSSION

Patients suffering from lepromatous leprosy show a depression in their ability to display several manifestations of the cell-mediated immune response. Lepromatous leprosy is recognized to be associated with profound anergy (absence of delayed type hypersensitivity) to antigens of *M. leprae* (Mitsuda, 1953). The ability of these patients to become sensitized to chemicals such as dinitrochlorobenzene (DNCB), 2-chloro-1,3,5-trinitrobenzene (TNCB), or picryl chloride is reported to be diminished (Waldorf *et al.*, 1966; Turk & Waters, 1969; Bullock, 1968), and a delayed rejection of skin homografts has been described by Job & Karat (see Hart & Rees, 1967).

Studies *in vitro* have also demonstrated a moderate but generalized impairment of CMI (Bullock & Fasal, 1971; Talwar *et al.*, 1972; Godal *et al.*, 1971). Godal's group (1971) concluded that lepromatous patients lack circulating lymphocytes specifically reactive to *M. leprae*, but this does not explain the more general impairment of CMI observed by others both *in vitro* and *in vivo*.

An impairment in CMI responses, including delayed rejection of skin homografts and depression or absence of contact sensitivity to chemicals, has also been demonstrated in mice infected with rat-leprosy bacillus (Ptak *et al.*, 1970).

The rather non-specific impairment of CMI has been related to the marked histological changes observed in the thymus and lymph nodes of patients (Turk & Waters, 1969; 1971) and mice (Ptak *et al.*, 1970) infected with human- or rat-leprosy bacilli, respectively. In both cases, a progressive depletion of lymphoid cells in the paracortical areas of lymph nodes has been established and Turk & Waters (1969) have considered that the non-specific loss of CMI may be secondary to the infiltration of the paracortical areas of lymph nodes with histiocytes, rather than a primary event leading to the development of the lepromatous state.

On the other hand, no reports have been published regarding any depression in the antibody-mediated immune response in human or murine leprosy. On the contrary many reports exist that indicate that patients with leprosy have a rather high reactivity against several antigens. For example, antibody responses were normal or raised against mycobacterial antigens (Ulrich *et al.*, 1969), mycobacterial antigens and typhoid-paratyphoid vaccine (Jha *et al.*, 1971), and against smallpox vaccine (Saha *et al.*, 1973) etc. The high incidence of autoantibodies (rheumatoid factor, antinuclear antibody, antithyroglobulin antibody, and others) has also been recognized (Shwe, 1972; Malaviya *et al.*, 1972) and the non-specific hypergammaglobulinaemia was established long ago (Mamba & Fujiwara, 1952; Hoxter, Batista & Vellini, 1950; Ishihara, 1950; Bonomo, Dammacco & Guillardi, 1969; Carteron, Fauran & Courmes, 1970 and many others).

There are some reports on the antibody response to SRBC and serum proteins in mice infected with murine leprosy. Ptak *et al.* (1970) studied the antibody response to bovine serum albumin (BSA) and to SRBC. They found no differences from normal mice. Abe *et al.* (1972) studied haemolysin production to SRBC and contact allergy with DNCB, and again found no significant difference between infected animals and controls.

We found a progressive decrease in the ability of mice with murine leprosy to respond to SRBC, both in terms of serum antibodies and plaque-forming cells. However, during the first 75 days after infection the titre of antibodies against SRBC was higher in the infected group than in the controls. This transitory hyper-reactivity may arise from overstimulation of the reticuloendothelial system by the moderate numbers of mycobacteria present which could function as a non-specific adjuvant. By contrast, no significant differences were observed during this period between normal and infected animals immunized

with HGG. After 75 days the titre of serum antibodies and the number of PFC per whole spleen began to decrease in the infected group to reach values similar to those obtained with normal unimmunized mice. Progressive depletion of lymphoid cells in thymus and lymph nodes of infected mice may explain the impairment of CMI (Ptak *et al.*, 1970) and may also account for this gradual depression in antibody-mediated immune response. A marked impairment in CMI without a proportional impairment in antibody-mediated immunity could only occur if the depression in CMI is quite specific, for instance due to a lack or disfunction of *M. leprae*- or *M. lepraemurium*-reactive lymphocytes.

As both SRBC and HGG are thymus-dependent antigens, we believe that a lack or disfunction of thymus-derived lymphocytes to which a central role in CMI has been ascribed in human leprosy (Godal *et al.*, 1971; Dwyer, Bullock & Fields, 1973; Lim *et al.*, 1974), could explain the depressed ability of infected mice to show adequate humoral immune responses to these antigens. The depletion of lymphocytes in thymus and paracortical areas of lymph nodes (Ptak *et al.*, 1970) and the impaired thoracic duct lymphocytes recirculation in mice or rats infected with *M. lepraemurium* (Bullock 1972) provide some evidence in support of this idea. Similar experiments with thymus-independent antigens (e.g. SSS-III, polyvinylpyrrolidone, *E. coli*'s lipopolysaccharide) already in progress will provide more information about this point.

It is also possible that the function of another cell type, the macrophage is impaired. After 1 month of infection, about 20% of the mouse peritoneal cell population contained acid-fast bacilli (2% of the total cell population contained more than 10 bacilli per cell) and the number of infected peritoneal cells increased to about 50% after 3·5 months of infection (about 20% of the cells contained more than ten bacilli per cell) (unpublished work). Whether or not the reduction in the number of uninfected peritoneal cells (95% of which are mononuclear) is related to the low response to SRBC remains to be established. It is possible that antigen-handling by macrophage is affected by the intracellular parasite.

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