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Cell-mediated immune response during experimental arthritis induced in rats with streptococcal cell walls

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SUMMARY

Chronic, remittent, erosive arthritis was produced in rats by a single intraperitoneal injection of an aqueous suspension of cell wall fragments isolated from group A streptococci. Arthritis could be induced in rats which had been immunologically compromised by neonatal thymectomy. Delayed hypersensitivity to cell wall peptidoglycan could not be elicited in these rats, although progressive joint disease was obvious by clinical and radiological measurements. A delayed skin test was elicited with peptidoglycan in non-thymectomized rats at 6 to 14 days after injection of low doses of cell wall fragments. Between 2 to 4 weeks after cell wall injection the skin test could not be elicited and these rats could not be sensitized again with peptidoglycan. After a high dose of cell wall the skin test could not be elicited at any time. These non-thymectomized rats which had been injected with cell walls remained hyporesponsive to peptidoglycan for at least 3 months. Lymphocytes from non-thymectomized cell wall-injected rats also showed a non-specific depression of lymphocyte response to phytohaemagglutinin *in vitro*, but this function was recovered between 2 to 4 weeks after cell wall injection. We conclude that cell-mediated immunity against bacterial cell wall antigens is not a pathogenetic factor in this experimental model of arthritis.

INTRODUCTION

Chronic, remittent polyarthritis is produced in rats by a single intraperitoneal or intravenous injection of a sterile aqueous suspension of cell wall fragments isolated from group A streptococci (Cromartie *et al.*, 1977). The disease evolves over a period of 4 to 6 months with repeated cycles of remission and exacerbation involving most joints of the feet, ankles, wrists and knees. The progressive injury, which leads to loss of function with extensive destruction of joint surfaces and subchondral bone, has been monitored by histological and radiological analyses (Clark *et al.*, 1979; Cromartie *et al.*, 1977).

This experimental model of erosive synovitis is an extension of our studies on chronic inflammation induced by bacterial cell wall debris which the host cannot eliminate effectively (Ohanian, Schwab & Cromartie, 1969; Schwab *et al.*, 1967). The essential cell wall structure is the covalently bound peptidoglycan–polysaccharide complex (Abdulla & Schwab, 1966; Schwab, Cromartie & Roberson, 1959). An outstanding property of cell wall isolated from group A streptococci is its resistance to biodegradation and consequent persistence in tissue (Ohanian & Schwab, 1967; Schwab & Ohanian, 1967). In addition, the isolated peptidoglycan moiety is toxic (Abdulla &

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Schwab, 1966; Heymer & Rietschell, 1977); it can activate the alternate complement pathway (Greenblatt, Boackle & Schwab, 1978); and it can stimulate macrophages to become cytotoxic (Smialowicz & Schwab, 1977).

Because the peptidoglycan and polysaccharide moieties of group A streptococcal cell walls are antigenic, analysis of the mechanisms of tissue injury must consider the role of specific immune responses against these structures. Therefore, we have measured delayed hypersensitivity and the evolution of joint disease after cell wall injection in normal rats and in rats compromised by neonatal thymectomy. The rationale is based upon observations that an adult rat, thymectomized at birth, has an impaired capacity to develop delayed hypersensitivity and other manifestations of cell-mediated immunity (Arnason *et al.*, 1962; McCullogh, 1976), although neonatal thymectomy does not completely deprive a rat of thymus-derived cells (McCullogh, 1976).

This paper reports our studies on: (i) the effect of neonatal thymectomy upon the experimental model of rheumatoid arthritis in the rat, and the influence of cell wall dose upon the effect of thymectomy; (ii) the relationship of joint disease to delayed hypersensitivity against peptidoglycan; and (iii) the development of cell-mediated immune hyporesponsiveness in non-thymectomized rats injected with cell wall fragments. Antibody responses to cell wall antigens are described in an accompanying paper (Greenblatt, Hunter & Schwab, 1980).

MATERIALS AND METHODS

Animals. Outbred, Sprague–Dawley rats were purchased from Zivic-Miller, Allison Park, Pennsylvania. Animals from this stock were bred in our animal facilities and neonatal thymectomy was performed within 24 hr of birth by splitting the sternum vertically and removing each lobe of the thymus by a combination of blunt dissection and suction. Littermates were matched for thymectomy or sham thymectomy. Males and females were included in both groups. Tetracycline (Lederle) was added to the drinking water of all rats (mothers and weaned rats) throughout the experiment in a concentration of 250 mg/l (estimated to be 8 $mg \cdot rat^{-1} \cdot day^{-1}$). When the animals reached an average weight of 150 g (about 7 weeks old) they were given one intraperitoneal (i.p.) injection with a sterile suspension of cell wall fragments in pH 7·2 phosphate-buffered saline (PBS).

Bacterial cells and cell walls. The details of these procedures have been described previously (Cromartie *et al.*, 1977; Ohanian & Schwab, 1967). Group A, type 3, strain D-58 streptococci were grown in Todd–Hewitt broth (DIFCO, Detroit, Michigan) for 16 hr at 37°C. The cells were harvested by centrifugation, washed and disrupted in a Braun shaker (Bronwell Scientific Co., Rochester, New York). Cell walls were collected by differential centrifugation, treated with trypsin and ribonuclease and washed five times. Cell wall fragments were prepared by suspending cell walls in pH 7·2 PBS and subjecting them to sonic treatment for 70 min in a Branson sonifier (Heat Systems–Ultrasonics, Plainview, New York). After sonication the walls were filtered through a millipore 0·45-μm filter (Millipore Corp., Bedford, Massachusetts) and the concentration of rhamnose, *N*-acetyl glucosamine and *N*-acetyl muramic acid measured by gas chromatography. Sterility was confirmed by culture on blood agar plates.

Design of experiments. This study was designed to obtain periodic assessment of joint disease by clinical and radiological measurements, skin test reactions to peptidoglycan, and *in vitro* response of lymph node cells to stimulation by phytohaemagglutinin (PHA, DIFCO Laboratories, Detroit, Michigan). All of these measurements were compared in neonatally thymectomized and non-thymectomized rats injected with different doses of cell wall fragments. Sixty-two thymectomized and thirty-five non-thymectomized rats were injected with cell wall fragments in a dose of 60 μg of rhamnose per g body weight. More thymectomized rats were injected because we anticipated a higher death rate in this group. A higher incidence of death, however, did not occur. At intervals of 3, 15, 30, 56 and 86 days after injection, randomly selected groups of five to ten thymectomized and five to ten non-thymectomized rats were skin tested, by intradermal (i.d.) injection on the flank, with 50 μg of peptidoglycan isolated from group A variant streptococcal cell walls (Abdulla & Schwab, 1966). The diameter and thickness of induration of the skin site were measured at 6, 24 and 48 hr after i.d. injection. The skin test score is the sum of thickness and induration in mm. These rats were

then bled and killed with ether. Four thymectomized and four non-thymectomized rats not injected with cell walls were included with each group. Aortic, inguinal and mesenteric lymph nodes were collected for tissue culture of lymphocytes in order to study the *in vitro* response to PHA.

The effect of cell wall dose upon the skin test reaction to peptidoglycan and joint lesion development in thymectomized and non-thymectomized rats were also studied. These measurements were done at intervals on forty-nine thymectomized rats and forty-one non-thymectomized rats injected with 20 μg of cell wall rhamnose per g of body weight, and on ten thymectomized and ten non-thymectomized rats injected with 5 μg of cell wall rhamnose per g of body weight. At the termination of all experiments all surviving rats were skin tested, bled and radiographed.

Evaluation of joint disease. All rats were observed daily during the first 3 weeks after cell wall injection and three to four times per week thereafter until termination of the experiments at 3 months. The severity was graded using a scale of 0 to 4 for each leg (maximum score 16). The score was based on the severity and extent of erythema and oedema of the periarticular tissues and the enlargement, distortion or ankylosis of the joints. The maximum acute joint lesion score was the greatest score recorded up to 16 days after cell wall injection (usually this score was achieved at 3 to 7 days after injection). The maximum chronic joint lesion score was the highest observed after 16 days following cell wall injection (usually between 20 to 60 days). Joint lesions were also assessed by X-ray and scored by a radiological technique described by Clark *et al.* (1979). The clinical and radiological evaluations were made by different individuals who were not aware of the other scoring results.

Evaluation of thymectomy. When rats were killed an estimate of gross thymic remnants was recorded. The tissue in the mediastinal region was fixed in formalin and serial histological sections prepared for microscopic examination. From the microscopic study 66% of the rats had no detectable thymus, 18% had 10% estimated thymus remnants, 9% had 20% thymus remnants and 6% had 30% thymus remaining. Animals with more than 10% thymus were not included in the calculations. In agreement with others (Arnason *et al.*, 1962) we did not see any consistent difference in measurements of thymic function between rats with 0 to 20% thymus remnants.

Thymic *function* was evaluated in the groups of thymectomized and normal rats which were killed at 3, 15, 30, 56 or 86 days after injection of cell walls. Thymectomized and normal control rats (not injected with cell walls) were included. Most of the remaining rats were also evaluated when they were killed at the termination of the experiments. A measurement of function was the response of lymph node cells to stimulation by PHA. Cell suspensions from cervical, axillary and inguinal lymph nodes were prepared in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated foetal calf serum, 25 mM HEPES buffer, 0·1% glutamine and 0·1 mg/ml of gentamicin. Cells were cultured in Falcon culture plates (3040) in a concentration of 1×10^6 cells per well. Cells from each animal were tested separately in quadruplicate with PHA in concentrations of 1, 5 or 10 μg per well. Cultures were incubated for 72 hr in 5% CO_2 at 37°C and ^{3}H-thymidine added for the last 12 hr of culture. Cells were harvested with a Mash II Cell Harvester and the incorporated radioactivity measured in a Packard liquid scintillation counter.

The net counts per min (c.p.m.) of lymphocytes from neonatally thymectomized control rats killed at each of the five intervals was 11 to 20% of the values of the normal control rats. As a further test of the thymectomy procedure, sixteen normal control rats and sixteen thymectomized control rats were sensitized to tuberculin by injecting a hind footpad with 0·05 ml of Freund's complete adjuvant (DIFCO) to which 6 mg/ml of additional *Mycobacterium butyricum* had been added. Twelve days later a delayed skin test reaction was elicited by i.d. injections of 250 units of PPD (purified protein derivative). The average diameter of induration at 24 hr in the normal group was 8·6 mm compared with 2·8 mm for the thymectomized group ($P < 0{\cdot}01$).

Delayed skin test reaction to peptidoglycan. The isolated peptidoglycan polymer is a dermonecrotoxin (Abdulla & Schwab, 1966) and therefore must be detoxified to an acceptable level to be used as an eliciting skin test antigen. This is done by reducing the polymer size by limited lysozyme digestion or sonication (Abdulla & Schwab, 1966).

Conditions of sonication optimal for reducing toxicity while retaining antigenicity were determined in preliminary experiments. For the present study 8·5 mg of peptidoglycan isolated from cell walls of group A-variant streptococci, strain K-43, were suspended in 17 ml of PBS and sonicated

for 20 min in a Branson sonifier using a 20-ml steel cup and a ½-inch probe. When injected intradermally into the flank of control rats in a dose of 50 μg an acute inflammatory reaction was produced which reached a peak response between 12 to 24 hr and then subsided. This reflects the residual toxicity of the peptidoglycan and all reactions were read against this background. When the peptidoglycan was injected intradermally into rats sensitized by either an i.p. injection of an aqueous suspension of cell walls, or by cell walls in Freund's complete adjuvant, a peak response was reached at 48 hr. Microscopic examination of biopsies of the skin test site showed histological features consistent with reactions to tuberculin in tuberculin-sensitized rats (Arnason *et al.*, 1962). On the basis of these observations we consider this a delayed hypersensitivity (DH) reaction to peptidoglycan. The skin reaction elicited with group A cell walls (peptidoglycan–polysaccharide complex) was not consistent, and the isolated group A polysaccharide elicited no reaction.

Normal control rats can also be sensitized by an i.d. injection of 50 μg of peptidoglycan. A delayed skin reaction, which reaches a peak in 48 hr, can be elicited by a second i.d. injection of 50 μg of peptidoglycan given within 6 to 9 days after the first injection. If rats are skin tested with a second or third i.d. injection of peptidoglycan after an interval greater than 12 days between injections, no reaction is elicited. This reaction resembles a cutaneous basophil hypersensitivity (CBH) or Jones–Mote reaction more than a DH reaction in the method of induction and the transient state of sensitivity (Dvorak, 1976). Unlike CBH in guinea-pigs, the reaction in rats has some induration and very few basophils. The important point, however, is that while CBH and DH may have distinguishing features, both are delayed cell-mediated immunological reactions (Dvorak, Colvin & Churchill, 1975).

RESULTS

Effect of cell wall dose and neonatal thymectomy on arthritis

The incidence and severity of joint disease was dose-related. Intraperitoneal injection of outbred Sprague–Dawley rats with 60 μg of cell wall rhamnose per g of body weight induced both the acute phase and the remittent, chronic phase of the disease in 100% of the animals. The incidence was 70%

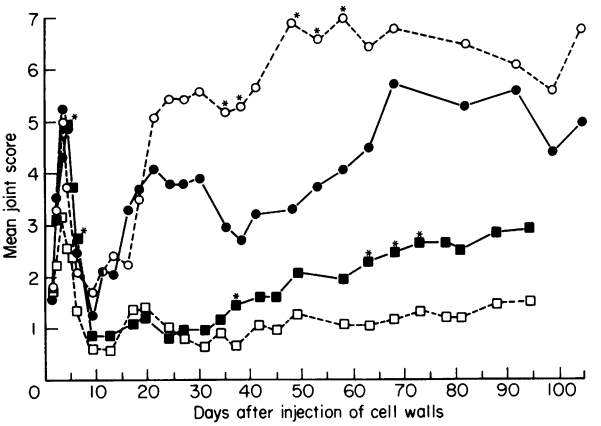

Fig. 1. The course of joint disease in neonatally thymectomized and non-thymectomized rats injected i.p. with an aqueous suspension of cell wall fragments from group A streptococci in a dose of either 20 or 60 μg of rhamnose per g body weight (●—●) Non-thymectomized and (o - - - o) thymectomized with 60-μg dose; (■—■) non-thymectomized and (□ - - - □) thymectomized with 20-μg dose. (*) Significant difference between thymectomized and non-thymectomized group at $P < 0{\cdot}05$. In the animals given a dose of 60 μg of cell wall rhamnose per g body weight there were initially sixty-two rats included in the thymectomized group, decreasing to twenty-eight as animals were removed for skin testing or histopathology; in the non-thymectomized group, there were initially thirty-five rats decreasing to fourteen. With the 20-μg dose there were forty-nine thymectomized decreasing to thirty-seven and forty-one non-thymectomized decreasing to thirty-five.

in rats injected with 20 μg of cell wall rhamnose per g body weight, and 10% in rats injected with 5 μg cell wall rhamnose per g body weight. Typically, there was an early acute inflammation of the joint which appeared within 1 to 2 days after cell wall injection and reached a peak at 3 to 5 days. This early reaction receded by 2 weeks and was followed by the chronic phase consisting of a series of remissions and exacerbations continuing for at least 3 months.

The effect of neonatal thymectomy on the clinical course of disease is shown in Fig. 1, and the terminal radiograph scores of the hind legs in Fig. 2. The correlation between clinical and radiograph scores was high ($r=0{\cdot}84$, $n=97$, $P<0{\cdot}0001$). It is evident that joint disease developed in thymectomized rats in spite of impaired T cell function. With a relatively high dose (60 μg of cell wall rhamnose per g) there was no difference in the acute phase of the disease over the first 2 weeks. As the disease progressed into the chronic, remittent phase the joint scores were greater in the thymectomized group at several intervals. However, after 60 days the difference between thymectomized and non-thymectomized rats was not significant (Fig. 1). This was confirmed by the terminal radiograph scores (Fig. 2).

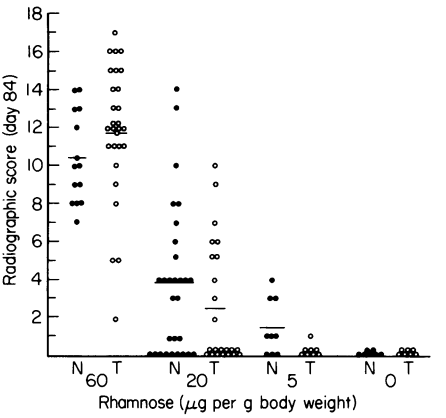

Fig. 2. Terminal radiographic scores of joint lesions in hind limbs of neonatally thymectomized (T) and non-thymectomized (N) rats injected with cell wall fragments. All rats surviving to day 86 are included. Each point represents one rat.

Thymectomized rats given a dose of 20 μg of cell wall rhamnose per g of body weight developed lower joint scores than non-thymectomized animals in both the acute and chronic phases, but because of the large variance, the difference between the groups was inconsistent and statistically significant at only a few intervals (Fig. 1). The terminal radiograph scores also failed to show a significant difference between these groups (Fig. 2). Injection of 5 μg of cell wall rhamnose per g body weight produced clinically apparent arthritis in one out of ten rats in both thymectomized and non-thymectomized groups. The incidence was higher in the thymectomized group by radiographic measurement, but the difference was not significant (Fig. 2).

Relationship of arthritis to delayed skin tests

From the non-thymectomized rats injected i.p. with cell walls in a dose of 60 μg of rhamnose per g body weight, groups of five to ten were randomly selected for skin testing with 50 μg of peptidoglycan at intervals after cell wall injection. In the group of rats injected with cell walls in a dose of 5 or 20 μg of rhamnose per g body weight, the same rats were skin tested at each time interval. The course of joint disease in these rats is depicted in Fig. 1. The skin test scores at 48 hr after peptidoglycan injection are shown in Fig. 3. The rats injected with cell wall doses of 5 or 20 μg of rhamnose per g body weight developed delayed reactions significantly different from controls ($P<0{\cdot}01$) when skin-tested with peptidoglycan for the first time at 7 days after cell wall injection. Thereafter the reaction was no greater than control rats not injected with cell wall. Rats given the high dose of cell wall (60 μg of rhamnose per g body weight) did not display any delayed reaction at any time, and in

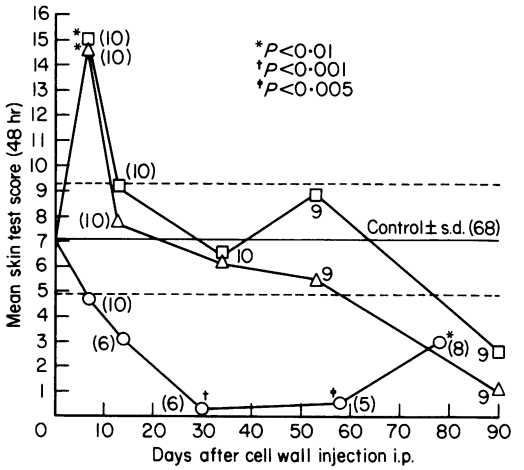

Fig. 3. Delayed skin test responses to peptidoglycan by non-thymectomized rats which had been injected i.p. with an aqueous suspension of cell wall fragments. Skin tests were elicited with 50 μg of peptidoglycan at intervals of 7 to 90 days after i.p. injection of cell walls in doses of 5 (□), 20 (△) or 60 (○) μg of rhamnose per g body weight. The number by each point is the number of rats skin tested in each group. Probability values are given for points which are significantly different from the control group skin-tested at the same interval. The horizontal lines indicate the mean skin test score ± 1 standard deviation for all control animals. The joint scores of the groups from which these rats were taken for skin testing are shown in Fig. 1.

Table 1. Anergy to peptidoglycan in non-thymectomized rats injected with cell wall fragments*

Group†	No. of i.d. injections	Interval between i.d. injections (days)	No. of rats	Skin test score (mm)‡	Level of significance (*P vs* control 1)
Control 1	2	7	32	16·4 ± 2·5	—
Cell wall (60 μg)	2	7	8	1·6 ± 0·8	< 0·001
Cell wall (20 μg)	2	7	5	6·2 ± 2·3	< 0·05
Cell wall (5 μg)	2	7	5	8·7 ± 2·8	n.s.
Control 2	1	—	68	7·0 ± 1·1	< 0·001
Control 3	2	21	10	7·2 ± 1·9	< 0·01
Control 4 (CW + FCA)	1	—	7	20·0 ± 2·9	n.s.
	2	21	7	20·8 ± 3·2	n.s.

* Anergy is measured by sensitization of rats for a delayed skin test reaction to peptidoglycan. Rats were sensitized by an i.d. injection of 50 μg of peptidoglycan. The reaction was elicited 7 days later with a second i.d. injection of 50 μg of peptidoglycan.

† Controls 1, 2 and 3 are normal rats not injected with cell wall; cell wall indicates rats injected i.p. 54 days earlier with cell wall fragments in doses of 60, 20 or 5 μg of rhamnose per g body weight; control 4 is the group of rats sensitized with cell wall in Freund's complete adjuvant and skin-tested with peptidoglycan 12 and 33 days latter (21-day interval between skin tests).

‡ Mean ± s.e. of induration at 48 hr.

fact the measurements were significantly below control rats when tested at intervals from 30 to 80 days after cell wall injection (Fig. 3).

In order to confirm that these cell wall-injected rats were anergic to peptidoglycan, we attempted to re-stimulate them for a delayed skin reaction as described in the Materials and Methods section. Rats were sensitized by an i.d. injection of peptidoglycan and the delayed reaction was elicited 7 days later with a second i.d. injection of peptidoglycan. In contrast to control rats, cell wall-injected rats were difficult to sensitize by this procedure and the degree of anergy was directly related to the dose of cell wall which had been injected (Table 1).

The neonatally thymectomized rats injected with the same doses of cell wall failed to develop delayed skin reactions at any time after cell wall injection, including the rats injected with the low doses of 5 or 20 μg of rhamnose per g body weight (Fig. 4). This is another criterion of the

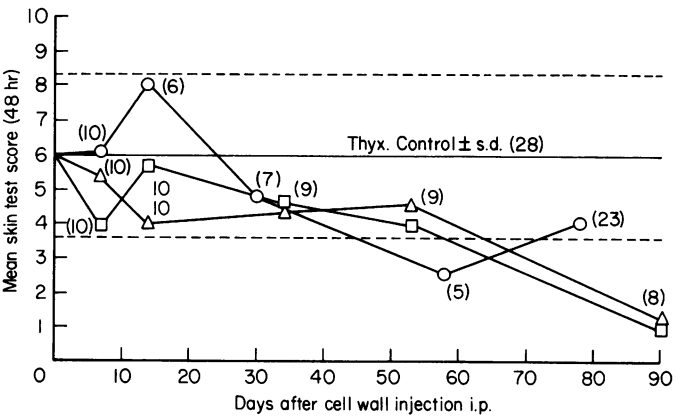

Fig. 4. Delayed skin test responses to peptidoglycan by neonatally thymectomized rats which had been injected with cell wall fragments in doses of 60 (○), 20 (△); or 5 (□) μg of rhamnose per g body weight. (See legend to Fig. 3 for key.)

Table 2. Effect of cell wall injection upon *in vitro* stimulation of rat lymph node cells by PHA

Days after cell wall injection	Group*	No. of rats	Net c.p.m.†
3	Control	4	5,564 ± 84
	Cell wall	6	469 ± 33
15	Control	4	5,521 ± 86
	Cell wall	6	1,145 ± 32
30	Control	4	8,177 ± 47
	Cell wall	5	8,498 ± 106
56	Control	5	5,404 ± 21
	Cell wall	5	8,688 ± 94
86	Control	14	10,914 ± 112
	Cell wall	13	11,562 ± 137

* Non-thymectomized rats, either not injected with cell wall (control) or injected i.p. with cell wall fragments in a dose of 60 μg of rhamnose per g body weight.

† Incorporation of ^{3}H-thymidine, counts per min (c.p.m.) in cultures with 5 μg of PHA, less c.p.m. in cultures without PHA.

effectiveness of the neonatal thymectomy. At the low cell wall doses the skin reactions at 7 days after cell wall injection were significantly less than those of non-thymectomized rats (compare Figs 3 and 4). It is noteworthy that the skin test responses of thymectomized rats given cell walls in a dose of 60 μg of rhamnose per g body weight were not significantly greater than the thymectomized controls but were greater than the comparable non-thymectomized rats given the same cell wall dose. This was consistently observed for 60 days after injection. There was no significant difference between thymectomized control rats (not injected with cell wall) and non-thymectomized control rats in the skin test responses (Figs 3 and 4). Regression analysis of maximum chronic joint lesion scores (achieved between 16 to 90 days after cell wall injection) and delayed skin tests in individual rats, measured at 7 days after injection of cell walls in a dose of 20 μg of rhamnose per g body weight, showed no correlation.

Effect of cell wall injection upon lymphocyte function

Lymph node cells from non-thymectomized rats injected with cell walls in a dose of 60 μg of rhamnose per g body weight had a reduced capacity to respond to PHA stimulation when removed 3 or 15 days after cell wall injection. This function returned to the normal level by 30 days (Table 2).

DISCUSSION

Both neonatally thymectomized and non-thymectomized rats injected i.p. with group A streptococcal cell wall fragments developed chronic, remittent, erosive arthritis in spite of impaired T cell function. The impairment of cell-mediated immunity was ascertained by a reduced mitogenic response of lymphocytes to PHA and absence of a delayed skin test reaction to peptidoglycan. We conclude that cell-mediated immune responses against bacterial cell wall antigens are not essential in the pathogenesis of this model of experimental arthritis.

Thymectomy of rats has been employed in the analysis of other models of experimental disease which are proposed to have a delayed hypersensitivity component. Arnason *et al.* (1962) reported that adjuvant arthritis was not influenced by neonatal thymectomy. Lennon & Byrd (1973) showed that experimental allergic encephalomyelitis was suppressed by neonatal thymectomy, but the incidence of adjuvant arthritis in the same rats was greatly increased. Somewhat analogous to our results is the report that thymectomy of rats at 4 weeks of age enhanced induction of adjuvant arthritis with high, but not with low, doses of wax D (Kayashima, Toshitaka & Onoue, 1976). It must be noted that the model reported here has several features which distinguish it from adjuvant arthritis, which is induced with cell wall structures in oil and has a different clinical course. Hadler & Granovetter (1978) have recently reviewed and compared the features of various models of arthritis induced with bacteria.

An interesting aspect of this study was the finding that non-thymectomized rats injected with cell walls also developed an impaired cellular immune capacity while the joint disease was evolving. The mechanism of this depressed immune function is being investigated. It could be related to the splenic suppressor macrophage described in rats by Oehler *et al.* (1977). We do not know if this hyporesponsiveness, or anergy, is a factor in the development of experimental arthritis. A deficient immune response could affect distribution or persistence of cell wall material. Hyporesponsiveness has also been reported in patients with rheumatoid arthritis (Andrianakos *et al.*, 1977; Waksman *et al.*, 1975).

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