

Modulation of the Susceptibility of Inbred and Outbred Rats to Arthritis Induced by Cell Walls of Group A Streptococci

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Inbred Buffalo rats were resistant to the induction of experimental arthritis induced by systemic injection of cell wall fragments in a crude whole-cell sonic extract of group A streptococci. This was in contrast to the susceptibility of outbred Sprague-Dawley and certain other inbred strains. Preliminary breeding studies indicated that genetic control of resistance of susceptibility is multigenic. When Buffalo rats were injected with a saline suspension of isolated cell wall fragments, chronic remittent arthritis developed. Suspension of the isolated cell walls in the supernatant fraction of group A streptococci solubilized by sonication eliminated the arthropathogenicity in Buffalo rats. Thus, a component separable from the cell wall fraction can modulate the arthropathogenicity of cell walls in rats, but the effect depends upon the genetic background of the rat. The antibody response of Buffalo rats to the polysaccharide antigen of cell walls was also affected by the supernatant fraction of sonicated group A streptococci.

Studies of the biological properties of components of streptococcal cells have permitted the development of several models of chronic inflammatory diseases in rabbits, mice, rats, and guinea pigs. Aqueous suspensions of peptidoglycan-C polysaccharide complexes of group A streptococcal cell walls will induce a chronic remittent and intermittent multinodular lesion in the skin of rabbits (14). A model of chronic pancarditis in mice (3) is associated with the localization and persistence of peptidoglycan-C polysaccharide complexes in the cardiac tissues after a single systemic injection of cell walls (11). Two models of chronic arthritis, one in rabbits (13) and one in rats (2, 4), are also associated with the persistence of group A streptococcal cell wall fragments in the inflamed joints. In guinea pigs, lesions of the heart, joints, and external ears are induced by a single intraperitoneal (i.p.) injection of cell wall fragments (W. J. Cromartie, J. H. Schwab, F. G. Dalldorf, and S. K. Anderle, *In M. T. Parker (ed.), VII International Symposium on Streptococci and Streptococcal Diseases*, Reedbooks, Chertsey, England, 1978, in press).

Inbred Buffalo rats, in contrast to outbred Sprague-Dawley and other inbred strains, are highly resistant to the induction of chronic arthritis when injected i.p. with an aqueous suspension of a crude cell wall preparation in the form of a whole-cell sonic extract of group A streptococci (S. K. Anderle and W. J. Cromartie, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1976,

B49, p. 19). This paper extends these observations and shows that Buffalo rats can develop chronic arthritis when injected with isolated cell wall fragments of group A streptococci. Evidence is presented that a soluble component in the supernatant of the whole-cell sonic extract of group A streptococci can modulate the susceptibility of rats to the arthropathogenic cell wall fragments, and this effect is dependent upon the genetic background of the animal.

MATERIALS AND METHODS

Animals. Inbred Buffalo, Lewis, and Fischer 344 rats and outbred Wistar rats were obtained from Simonsen Laboratories, Bilroy, Calif.; Long Evans outbred rats were purchased from Blue Spruce Labs, Altamont, N.Y.; and outbred Sprague-Dawley rats were from Zivic-Miller, Allison Park, Pa. Female rats weighing approximately 100 g each were used in all experiments, except as noted.

Bacterial cultures. The culturing of group A, type 3, strain D58 streptococci and the preparation of a sterile extract of sonically disrupted cells have been described (4). A lyophilized culture was inoculated into 10 ml of Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 6 h at 37°C. This was inoculated into 2.4 liters of broth, and after 18 h the cells were centrifuged at 7,000 rpm (8,000 × g) in the GSA rotor for 20 min at 4°C in the RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The cells were washed three times with sterile phosphate-buffered saline (PBS; pH 7.0).

Whole-cell sonic extract. The washed, packed cells were suspended in 35 ml of PBS, then subjected

to 90 min of ultrasonic vibration in a Branson Sonifier (Model S125, Heat Systems Co., New York) at maximal power. The steel chamber was held in an ice bath, and water at 4°C was circulated through the jacket of the chamber. Sonication was stopped for 5 min at 20-min intervals to further reduce excessive temperature increase. The disrupted cell material was sterilized by filtration through a series of membrane filters (Millipore Corp., Bedford, Mass.), 3.0, 1.2, and 0.45 μm in succession. Sterility was tested by inoculating 0.1 ml of filtered sonic extract on a sheep blood agar plate. Rhamnose content of the cell wall material was determined by the method of Dische and Shettles (5).

Supernatant from whole-cell sonic extract. Whole-cell sonic extract was centrifuged at $120,000 \times g$ for 2 h, and the supernatant was removed. The rhamnose content of the supernatant was determined, and the amount of rhamnose in the supernatant was included in the calculation of the total dose of cell wall material injected when purified cell wall fragments were suspended in the supernatant of the whole-cell sonic extract. Of the rhamnose, 92% was removed from the sonic extract by this centrifugation.

Purified cell wall fragments. The washed, packed cells were suspended to approximately a 15% suspension (wet wt/vol) in pH 7.0 PBS. A 30-ml amount was added to 30 g of glass beads (0.1/0.11 mm, VWR Scientific, San Francisco). One drop of tributyl phosphate was added, and the cells were broken by treatment in a Braun shaker (Bronwill Scientific Inc., Rochester, N.Y.) for 3 min with flowing CO_2 to keep the temperature close to freezing. The beads were allowed to settle, and the extract was centrifuged at $10,000 \times g$ for 30 min. The top layer of sediments was carefully washed off from a lower layer of "unbroken" cells with PBS. The top layer of cell walls was washed four times with PBS with complete dispersion obtained in each suspension by treatment in a Raytheon 9Kc sonic oscillator for 30 s. They were then washed three times with deionized water and lyophilized. This is hereafter referred to as the crude cell wall preparation. Intact cells could not be detected by Gram stain or phase microscopy. The crude cell wall preparation was suspended in PBS (10 mg/ml) and treated with 0.025% ribonuclease (0.025 mg/mg of cell wall) at 37°C for 4 h. They were washed once with PBS and treated with 0.025% trypsin at 37°C for 4 h. After they were washed two times with PBS and three times with deionized water, they were lyophilized. This preparation is hereafter referred to as purified cell walls. All procedures were conducted aseptically, and the stock enzyme solutions were filtered through a 0.22- μm Millipore filter before being added to the cell walls. To prepare small fragments of cell walls, the lyophilized purified cell walls were suspended in 40 ml of PBS (20 mg/ml) and subjected to ultrasonic vibration for 70 min, as described above, and then filtered through 1.2- and 0.45- μm sterile Millipore filters. The rhamnose value was then determined on the sample. This preparation is hereafter referred to as purified cell wall fragments. Sterility was confirmed by culturing 0.1 ml on sheep blood agar plates.

Method of scoring the arthritis. Rats were examined two or more times during day 1 after inoculation and then daily for the duration of each study. The severity of the disease was graded on a scale of 0 to 4

for each extremity. The score was based on the number of joints involved, the severity and extent of the erythema and edema of the periarticular tissues, and the enlargement, distortion, or ankylosis of the joints. The method used is similar to that developed by Wood et al. (18) for scoring adjuvant arthritis, with the exception that lesions of the skin or tails did not occur in the model under study. The maximum total score that a rat might receive in our system is 16. The joints scored were those of each extremity distal to the knees and elbows. Joint lesions were also assessed by X ray and scored by a radiological technique described by Clark et al. (2).

Histological studies. Selected animals were sacrificed at various intervals after inoculation. Specimens were fixed in 10% Formalin, and paraffin sections were stained with Weigert's hematoxylin and alcoholic eosin. The joint specimens were decalcified in formic acid-sodium citrate solution after fixation for 5 to 14 days.

Breeding of rats. Rats were bred in our animal facilities. Both male and female offspring were used. No effect of sex on the response was observed.

Statistical analyses. Data were analyzed by Student's *t* test and one-way analysis of variance.

Antibody measurement. Antibodies specific for the peptidoglycan and polysaccharide antigens of group A streptococcal cell walls were measured by a radioimmunoassay technique (N. Hunter, J. J. Greenblatt, S. K. Anderle, R. R. Brown, R. L. Clark, W. J. Cromartie, and J. H. Schwab, submitted for publication). ^{125}I is conjugated to isolated peptidoglycan or polysaccharide by first tyrosylating these structures (8). The methods described by Heymer et al. (8) and Bernstein, Klapper, and Krause (1) were adapted to rat serum. The labeled peptidoglycan bound to antibody was precipitated by polyethyleneglycol, and the labeled polysaccharide bound to antibody was precipitated by 50% saturated $(\text{NH}_4)_2\text{SO}_4$. ^{22}Na was used as a volume marker (6).

RESULTS

Comparative susceptibility of rat strains to arthritis induced by whole-cell sonic extract of group A streptococci. Our study of the comparative susceptibility of rat strains to arthritis induced by whole-cell sonic extract of group A streptococci is summarized in Table 1. Each animal received an i.p. injection of whole-cell sonic extract containing a cell wall dose of 60 μg of rhamnose per g of body weight. The animals were observed daily over a period of 19 days for development of joint inflammation. None of the Buffalo rats developed arthritis. Of the 12 Fischer 344 rats, 7 developed arthritis, and all of the animals of the other strains developed the disease. The severity and course of the disease observed in the Sprague-Dawley, Long Evans, and Wistar rats was the same as that previously reported in the Sprague-Dawley strain (4). The severity of the joint reactions in the Fisher 344 strain was less than that previously described in Sprague-Dawley rats.

TABLE 1. Susceptibility of rats to the arthropathic property of whole-cell sonic extract of group A streptococci

Rat strain	Incidence ^a
Sprague-Dawley	12/12
Long Evans	12/12
Wistar	12/12
Lewis	6/6
Fischer 344	7/12
Buffalo	0/12

^a Each value indicates number of rats that developed arthritis per total, within 19 days after a cell wall dose of 60 μ g of rhamnose per g i.p.

This experiment was repeated, using doses of whole-cell sonic extract containing cell wall concentrations of 60, 80, or 100 μ g of rhamnose per g (Table 2). All of the Sprague-Dawley rats developed arthritis. Of the 24 Fischer 344 rats, 14 developed the disease. None of the Buffalo strain developed arthritis over an observation period of 122 days, with any dose given.

Modification of susceptibility by cross-breeding. Results of preliminary breeding studies are shown in Table 3. All rats were injected i.p. at approximately 6 to 8 weeks old (100 g) with crude-cell sonic extract of group A streptococci in a dose of 60 μ g of rhamnose per g. Animals were observed over a period of 65 to 127 days. The data show that susceptibility (or resistance) is under genetic control, and this is probably polygenic. The susceptibility of Sprague-Dawley and the resistance of Buffalo rats are not the result of single dominant genes. In the Fischer \times Buffalo crosses, the gene or genes determining resistance are dominant, since none of these rats developed any macroscopically detectable joint inflammation.

Susceptibility of buffalo rats to arthritis induced by purified cell walls. Investigation of the genetic control of resistance of Buffalo rats was pursued by injection of the less complex purified group A streptococcal cell walls. These had been separated from cytoplasmic components and consisted essentially of covalently bound peptidoglycan-polysaccharide. The arthropathogenic response of Buffalo rats to purified cell walls was dramatically different from the response to an equivalent dose of cell wall in crude sonic extract (Fig. 1). In both preparations, the cell walls had been subjected to comparable sonication, and each group of rats was injected i.p. with a dose of 60 μ g of rhamnose per g. All of the 16 rats injected with purified cell walls suspended in PBS developed chronic remittent arthritis which continued for at least 100 days, whereas none of the 24 rats injected with whole-

TABLE 2. Effects of dose of whole-cell sonic extract of group A streptococci on articular reaction in three strains of rats

Strain	Dose (μ g of rhamnose/g)	Avg score of arthritis at day 3	Incidence ^a
Sprague-Dawley	60	5.4	28/28
	80	8.1	23/23
Fischer 344	60	1.0	6/12
	80	4.9	8/12
Buffalo	60	0	0/57
	80	0	0/22
	100	0	0/4

^a Each value indicates number of rats that developed arthritis per total. All rats were observed for a period of 98 to 122 days after i.p. injection.

TABLE 3. Effect of cross-breeding on susceptibility of rats to arthritis induced with whole-cell sonic extract of group A streptococci^a

Group ^b	No. positive/total	% Positive	Days observed
(BUF \times SD)F ₁	22/80	27.5	127
(SD \times BUF)F ₁	15/51	29.4	127
(BUF \times SD)F ₂	7/28	25.0	68
(SD \times BUF)F ₂	4/18	22.2	69
SD \times (SD \times BUF)BC	4/14	28.5	65
(F344 \times BUF)F ₁	0/22	0	65
(BUF \times F344)F ₁	0/18	0	67
(F344 \times BUF)F ₂	0/9	0	69
(BUF \times F344)F ₂	0/8	0	67

^a Dose was 60 μ g of rhamnose per g i.p.

^b BUF, Buffalo strain; SD, Sprague-Dawley strain; F344, Fischer strain; BC, backcross; F₁, first generation of cross-breeding; F₂, offspring of brother-sister mating of F₁.

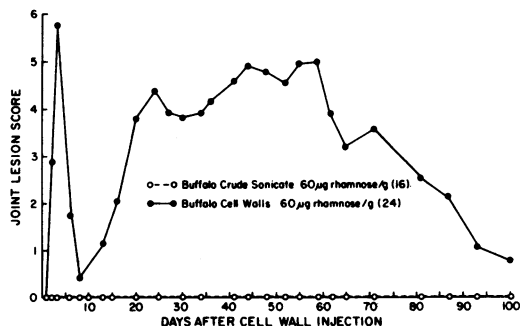


FIG. 1. Course of arthritis (mean of joint lesion score) induced in 24 Buffalo rats given a single i.p. injection of purified cell wall fragments suspended in PBS, compared with 16 Buffalo rats injected with a crude whole-cell sonic extract of group A streptococci. Each animal received a dose of 60 μ g of rhamnose per g.

cell sonic extract developed macroscopically detectable disease.

A comparison of the arthropathic response of Sprague-Dawley and Buffalo rats to purified cell walls in doses of 60 or 20 μg of rhamnose per g is shown in Fig. 2. Buffalo rats were quantitatively less susceptible to the 20- μg dose throughout the experiment. This difference did not become apparent with the high dose until the late chronic, remittent phase of the disease, i.e., past 60 days after injection of cell walls.

Modulation of arthropathogenic response to cell walls by the supernatant fraction of a sonic extract of group A streptococci. The arthropathogenicity of three cell wall preparations was compared in Buffalo (Fig. 3 and 5) and Sprague-Dawley (Fig. 4) rats. These three preparations were: (i) whole-cell sonic extract; (ii) purified cell walls suspended in the supernatant of whole-cell sonic extract (i.e., solubilized cell wall, cytoplasmic, and membrane components); and (iii) purified cell walls suspended in PBS. Rats in each group were injected with a cell wall dose of 60 μg of rhamnose per g. Joint lesion scores were significantly greater at all time intervals in Buffalo rats given cell walls in PBS compared with cell walls injected as crude whole-cell sonic extract or cell walls injected with the supernatant of the whole-cell sonic extract (Fig. 3). In the Sprague-Dawley rats, cell wall suspended in sonic supernatant induced the highest joint scores over the first 18

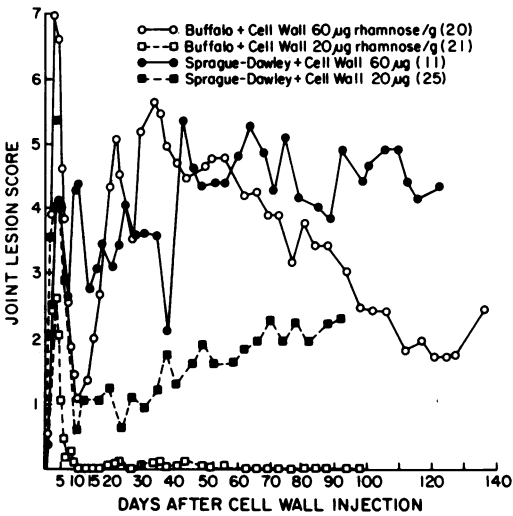


FIG. 2. Comparison of the course of arthritis in Buffalo and Sprague-Dawley rats injected i.p. with purified group A streptococcal cell wall fragments suspended in PBS in a dose of 60 or 20 μg of rhamnose per g. Each number in parentheses indicates the number of rats per group.

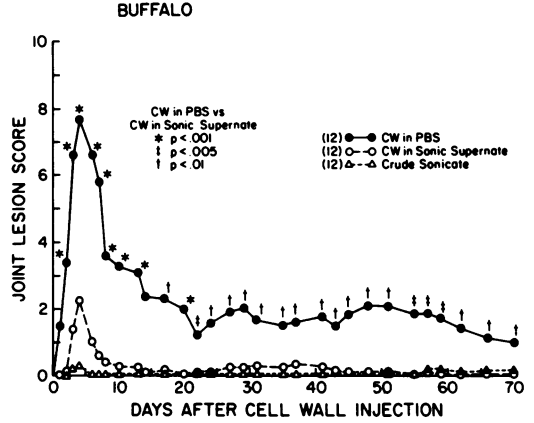


FIG. 3. Comparison of arthritis (mean of joint lesion score) induced in Buffalo rats injected i.p. with: purified cell walls from group A streptococci suspended in PBS; purified cell walls suspended in supernatant of a crude sonic extract of group A streptococci; or crude whole-cell sonic extract. Each point represents the mean of results from 12 rats. Each animal received a dose of 60 μg of rhamnose per g.

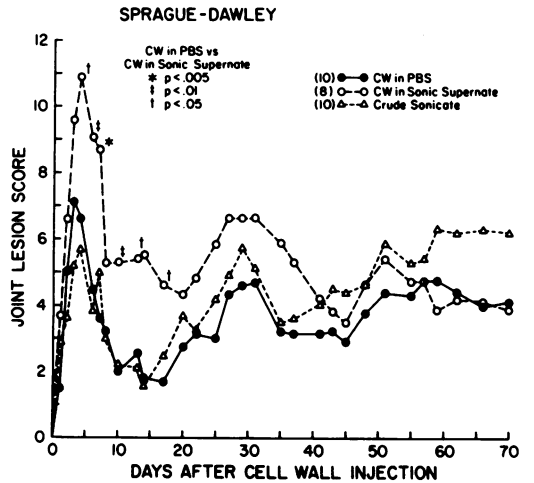


FIG. 4. Comparison of arthritis (mean of joint lesion scores) in Sprague-Dawley rats injected i.p. with: purified cell walls from group A streptococci suspended in PBS; purified cell walls suspended in supernatant of a crude sonic extract of group A streptococci; or crude sonic extract. Each number in parentheses indicates number of rats per group. Each animal received a dose of 60 μg of rhamnose per g.

days, but there was no significant difference between groups during the remaining 52 days of the experiment (Fig. 4).

Comparison of the three preparations in terms of incidence of arthritis shows the same relative effectiveness in Buffalo rats (Fig. 5) as shown by the quantitative assessment of joint scores illus-

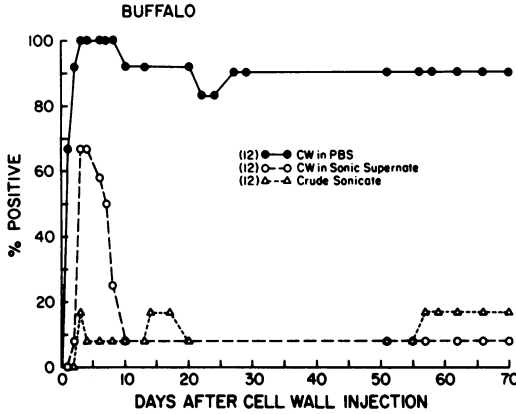


FIG. 5. Incidence of arthritis in Buffalo rats. These are the same groups for which the joint lesion score is given in Fig. 3.

trated in Fig. 3. In the Sprague-Dawley rats, all of the animals in each group developed arthritis.

The radiographic scores, recorded by a different group of observers (Fig. 6), demonstrate the same relative effect of the three cell wall preparations in Buffalo and Sprague-Dawley rats as was shown by the macroscopic joint scores illustrated in Fig. 3 and 4. Among the Buffalo rats, the score of the group given cell wall in PBS is different from the other groups at a level of significance of $P < 0.001$. Each group of the Sprague-Dawley rats is different from the Buffalo rats at a level of $P < 0.001$.

Histological studies. A complete histological analysis of the disease induced in the Buffalo strain has not been done. Sections from selected animals which had active arthritis at the time the experiments were terminated have shown erosive synovitis similar to that observed in the Sprague-Dawley strain (4). The animals studied were those injected with purified cell wall fragments suspended in PBS.

Antibody measurement. The antibody responses to the polysaccharide and peptidoglycan antigens of group A streptococcal cell walls reaches a peak in serum at 1 or 2 weeks after injection and declines to background levels by 5 to 6 weeks. The anti-polysaccharide levels of each group of Sprague-Dawley and Buffalo rats are shown in Fig. 7. The differences between groups of Sprague-Dawley rats is not significant because of the large individual variation within groups. This variation is a common feature of the response of outbred Sprague-Dawley rats to streptococcal cell wall antigens (10). It is not due to technical error, because agreement between replicate tests of one serum sample is very good. Nevertheless, it is clear that all groups of Sprague-Dawley rats produce 10- to 50-fold more anti-polysaccharide than Buffalo rats do

(Fig. 7). Among the Buffalo rats, the antibody response of the group given cell walls suspended in PBS is significantly greater ($P < 0.02$) than the other groups at 2 weeks after injection.

There were no significant differences between the rat strains or among groups in the anti-peptidoglycan antibody responses.

Included in Fig. 7 is the antibody response of two to four animals in the control groups of rats injected with only the supernatant from the whole-cell sonic extract. About 8% of the cell wall (i.e., rhamnose) remains in this fraction. Each of these rats received 9 μ g of rhamnose per g, which is sufficient to induce antibody response, but none of the rats injected with this fraction developed clinically or radiographically detectable chronic joint lesions.

DISCUSSION

These data demonstrate that the susceptibility or resistance of rats to experimental arthritis induced by bacterial cell walls is under genetic control. This control is multigenic, but further studies are necessary to determine how many genes are involved and whether this is associated with the major histocompatibility region.

It is also clear that the genetic control of response to cell walls, whatever its nature, can be modified by another component solubilized by sonication of group A streptococci. This is shown by the arthropathic response of Buffalo rats injected with isolated cell walls, and the reversal of this response if cell walls are mixed back with the supernatant fraction of the whole-cell sonic extract before injection. In the Sprague-Dawley rats, the sonic supernatant fraction had the opposite effect on the arthropathogenicity of cell walls over the early phase of joint inflammation, and thereafter there were no significant differences between the groups of this outbred strain.

These studies do not clarify the mechanism(s) of the genetic control, or of the modifying effect(s) of the supernatant fraction, which determine the arthropathic response to cell wall fragments. The Buffalo rats do produce much less antibody to the cell wall polysaccharide component than do Sprague-Dawley rats, and the injection of supernatant fraction with cell walls does significantly suppress the antibody response of Buffalo rats to the polysaccharide. However, other studies (N. Hunter et al., submitted for publication) fail to show any correlation between joint lesion scores and anti-polysaccharide responses in individual animals. In addition, we have not measured the response of other inbred rat strains and hence do not know whether this reduced response is peculiar to Buffalo rats. Thus, the relevance of this differ-

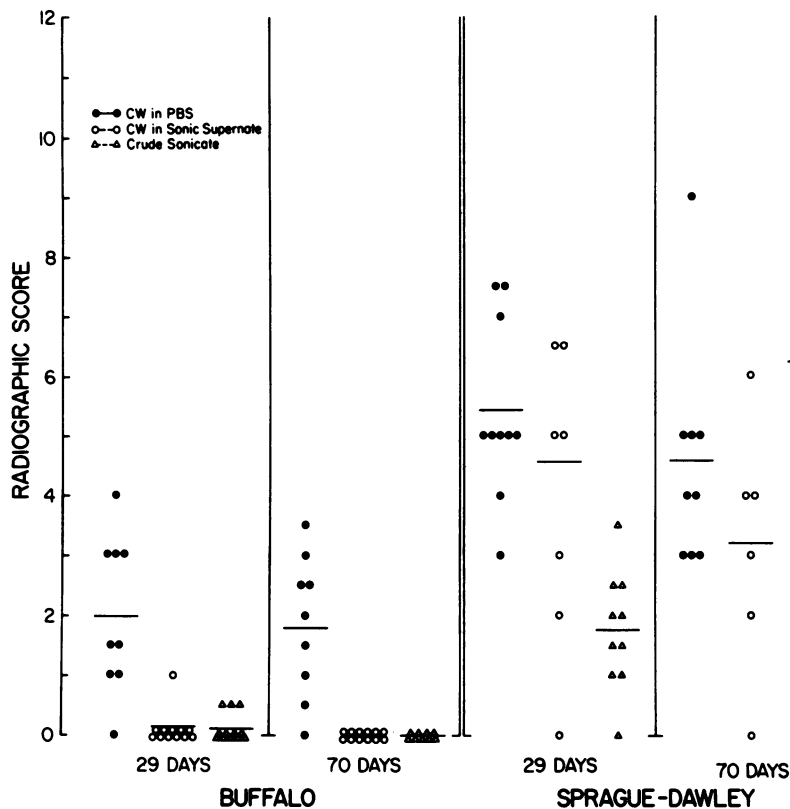


FIG. 6. Radiographic scores of arthritis in Buffalo and Sprague-Dawley rats. These are the same groups for which the macroscopic joint lesion score is recorded in Fig. 3 and 4. Each point is the score of one rat X-rayed at 29 and 70 days after an i.p. injection of one of the three cell wall preparations in a dose of 60 μg of rhamnose per g. Bars indicate mean score of each group. See text for levels of confidence.

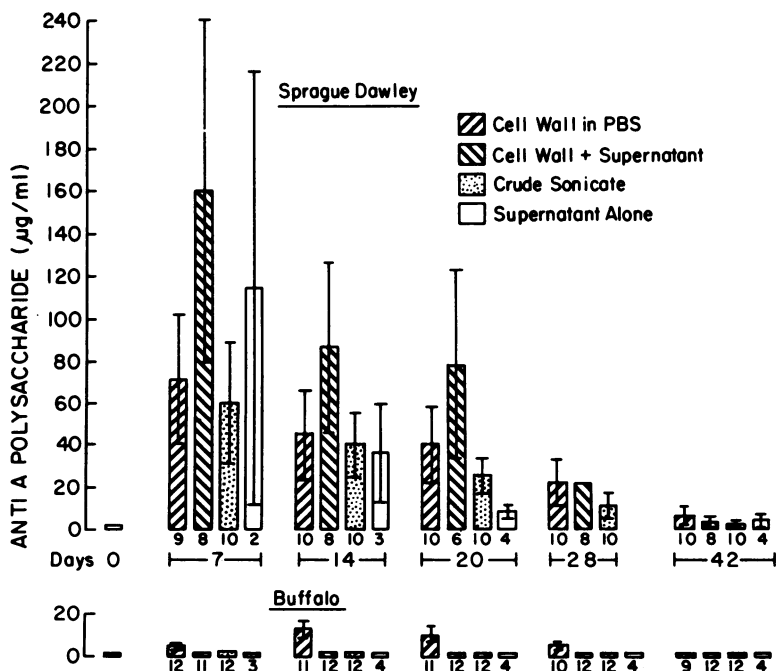


FIG. 7. Comparison of the serum concentration of antibody against the polysaccharide antigen of group A streptococcal cell wall in Buffalo and Sprague-Dawley rats. Bars indicate ± 1 standard deviation. Numbers below columns indicate number of rat sera measured. Rats were bled before injection and at 7, 14, 28, and 42 days after injection.

ence to the pathogenesis of joint disease remains uncertain.

Another possible mechanism of genetic control could involve distribution and persistence of cell wall material which, in part, reflects macrophage function. In vivo measurement of the fate of cell wall structures is in progress. We have previously described the processing of ¹⁴C-labeled streptococcal cell walls by Buffalo and Fischer 344 peritoneal macrophages in tissue culture (16). Under these in vitro conditions (16), there was no difference between strains in the rate of phagocytosis or degradation of group A streptococcal cell walls. The only consistent difference was the increased degradation of group D streptococcal cell walls by Buffalo macrophages.

Buffalo rats display other distinctive features, including spontaneous autoimmune thyroiditis which is enhanced by neonatal thymectomy (15). There are several characteristics which distinguish the model of experimental arthritis described in this report from adjuvant arthritis (7); but in that disease Buffalo rats have also been shown to have low susceptibility, whereas the Lewis strain is highly susceptible and the Fischer 344 strain has intermediate susceptibility (9). Susceptibility to adjuvant arthritis was reported to be inherited as a dominant trait (9).

The active component present in the supernatant fraction of the whole-cell sonic extract has not been identified. Work is in progress to determine whether the component is derived from the cytoplasm or cytoplasmic membrane or is a solubilized structure from the cell wall. Earlier studies, utilizing the model of chronic remittent skin lesions in rabbits, also showed that the reaction to streptococcal cell walls could be modified by a cytoplasmic fraction injected with the cell walls (12). Those observations, and our recent studies on immunosuppression and modification of lymphocyte functions in mice (17), suggest that the cytoplasmic membrane is a possible source of this activity.

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