INFECTION AND IMMUNITY, Oct. 1982, p. 127–135
0019-9567/82/100127-09$02.00/0

Vol. 38, No. 1

Measurement of Bacterial Cell Wall in Tissues by Solid-Phase Radioimmunoassay: Correlation of Distribution and Persistence with Experimental Arthritis in Rats

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Received 6 April 1982/Accepted 12 June 1982

We have developed sensitive and specific solid-phase radioimmunoassays to quantitate the distribution and persistence of bacterial antigen in rats developing arthritis in response to a single injection of streptococcal cell wall material. Three separate assays were specific for either the A polysaccharide (*N*-acetyl-D-glucosamine), A-variant polysaccharide (polyrhamnose), or peptidoglycan (D-ala-D-ala) moieties of the streptococcal cell wall. Antigen was detected in all tissues surveyed, although the greatest amount was in the liver and spleen. By using three fractions of cell wall separated by size, we have shown that the development of arthritis correlates with the degree of cell wall deposited and persisting in the joints. Further statistical analyses suggested differences in metabolism by different tissues and differential metabolism of different antigenic epitopes in some cases.

A single intraperitoneal injection of an aqueous suspension of purified group A streptococcal cell walls induces a chronic, progressive, destructive polyarthritis in rats (2, 4, 5). The disease is characterized by erosive synovitis of the small joints of the fore- and hindlegs and follows a clinical course of exacerbations and remissions that resembles human rheumatoid arthritis.

Our current understanding of the pathogenic mechanisms involved in this animal model is as follows. The purified cell wall consists of covalently bound polymers of peptidoglycan and group-specific polysaccharide. The peptidoglycan moiety has the capability of inducing inflammation (1), complement activation (8), and macrophage activation (22). In addition, the cell wall is relatively resistant to degradation in vivo, probably because of the protective nature of the group A polysaccharide (1, 19, 20). Immunofluorescent studies of tissues taken from arthritic rats document the persistence of cell wall antigens (4, 5). Although dysfunction of several facets of the immune system accompanies the development of joint disease (described by J. H. Schwab *in* S. Koatani, ed., *Immunomodulation by Microbial Products and Related Synthetic Compounds*, in press), as yet no evidence directly implicates either cell-mediated immunity or antibodies specific for cell wall antigens in the mechanisms of tissue injury (9, 11).

To investigate quantitatively the relationship of persistent streptococcal cell wall material to the occurrence of arthritis, we have developed highly sensitive and specific radioimmunoassays (RIAs) that detect cell wall antigens and have applied them to tissues from arthritic rats. An earlier report (6) described the different clinical patterns of joint disease produced with cell wall fragments of different size. In this paper, the quantitative assay is used to determine whether these variations in the severity of acute and chronic phases of joint inflammation, induced with fragments of different size, can be related to differences in tissue distribution and rate of elimination. The results confirm that cell wall material indeed persists, although it is slowly catabolized over time. In addition, the severity of arthritis correlates with the amount of cell wall material present in the joint.

(A preliminary account of this work has appeared in abstract form [Arthritis Rheum. 24(Suppl.):S64, 1981].)

MATERIALS AND METHODS

Induction and evaluation of arthritis. Outbred Sprague-Dawley rats were purchased from Zivic-Miller, Allison Park, Pa. Rats (8 weeks old), weighing

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approximately 100 g, were injected intraperitoneally with a single dose of sterile cell wall fragments (30 μg of rhamnose per g of body weight or about 10 mg of cell wall per rat) suspended in 0.1 M phosphate-buffered saline (pH 7.2). Control rats were injected with phosphate-buffered saline alone. Rats were housed two to a cage and observed for clinical evidence of arthritis daily over the first 2 weeks and 3 times a week thereafter. The severity of joint disease in each limb was estimated on a scale of one to four based on the degree of swelling, redness, and deformity (4).

Bacterial cell walls. A detailed description has been presented previously (6). Briefly, group A, type 3, strain D58 streptococci were processed by a combination of disruption, centrifugation, enzyme digestion, solvent extraction, and sonication. The resulting material was separated into three sizes of fractions by differential centrifugation: 10P (10,000 × *g* pellet; average molecular weight, 500×10^6); 100P (100,000 × *g* pellet; average molecular weight, 50×10^6); and 100S (100,000 × *g* supernatant; average molecular weight, 5.3×10^6) (6).

Processing of tissues. At 4, 29, and 65 days after cell wall injection, groups of rats were killed by ether anesthesia. The wrist and ankle joints, lungs, kidneys, heart, spleen, liver, and blood were then harvested and weighed in an aseptic manner. Skin was removed from the joints, and the carpi or tarsi and surrounding subcutaneous tissue were processed. All tissues were kept frozen until extraction. For use in the RIA, the tissues were suspended in 0.2 M Tris buffer (pH 8.0) and homogenized individually in a Sorval Omnimixer (E. I. duPont de Nemours & Co. Inc., Rockville, Md.) for 15 min, with careful washing of the equipment between samples. Samples were further treated by heating to 100°C for 30 min in 0.2 M Tris buffer (pH 8.0) with 0.4% Nonidet P-40. Samples were again frozen until assay time. Immediately before assay, they were centrifuged at 1,000 rpm for 5 min to remove tissue fragments. Samples of 24-h urine were collected over the first 8 days after cell wall injection.

Antibodies. Rabbits were immunized with multiple intravenous injections of group A streptococcal or group A-variant streptococcal heat-killed, pepsin-treated, whole cell vaccine. Three antibody specificities were affinity purified from the resultant antisera for use in RIAs. Anti-group A polysaccharide antibody (anti-*N*-acetyl-D-glucosamine [NADG]) was affinity purified from anti-group A antiserum on a column of *p*-aminophenyl-*N*-acetyl glucosamine bound to epoxy-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.). Anti-A-variant polysaccharide was affinity purified from anti-A-variant antiserum on a column of purified A-variant polysaccharide bound to the epoxy-activated Sepharose. It was further absorbed with columns of D-ala-D-ala-D-ala (Vega-Fox Biochemicals Div., Tucson, Ariz.) and *p*-aminophenyl-NADG. Anti-peptidoglycan (anti-D-ala-D-ala) antibody was affinity purified from anti-A-variant antiserum on a column of D-ala-D-ala-D-ala bound to cyanogen bromide-activated Sepharose. $F(ab')_2$ fragments of each of these affinity-purified antibodies were prepared by pepsin digestion, and undigested intact immunoglobulin G (IgG) or pFc' fragments were thoroughly removed by a combination of gel filtration (G150, Pharmacia Fine Chemicals) and absorption with columns made of staphylococcal protein A (Pharmacia Fine Chemicals) and sheep anti-rabbit IgG Fc fragment. Sheep anti-rabbit IgG Fc was prepared by extensive absorption of sheep anti-rabbit IgG on a column of rabbit $F(ab')_2$ fragments. This absorbed antisera was then affinity purified on a rabbit IgG column.

RIAs. The streptococcal cell wall material in rat tissues was quantitated by three separate RIAs, using each of the anti-cell wall specificities. Polyvinyl flexible plastic 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl per well of 1 μg of the $F(ab')_2$ fragment of anti-cell wall antibodies per ml in 0.2 M Tris with 0.01% azide (pH 8.0) for 5 h at room temperature or overnight at 4°C. Unbound material was then aspirated, and the plates were nonspecifically coated with 200 μl of 0.5% sterile human serum albumin in Tris buffer with 0.4% Tween 80 for 1 h. The coating solution was removed, and tissue samples, which were diluted in the coating buffer, were added for incubation overnight at 4°C. The next day, intact IgG antibody of the same cell wall specificity was added in coating buffer at a concentration of 5 μg/ml. After 3 h of incubation at 4°C, the plates were again washed, and radioiodinated affinity-purified sheep anti-rabbit Fc fragment (20,000 to 80,000 cpm/ng) was added for a further 2-h incubation. After a final washing, the plates were dried, and the wells were cut out for counting in an automatic gamma scintillation counter (Searle Radiographics Inc., Chicago, Ill.). Counts were recorded on teletype tape, and data were processed by computer. Samples from a given tissue from all rats at all time points were run in a single assay, along with separate standard curves made with the 10P, 100P, and 100S cell wall preparations. As it was found that the assays detect the 100S preparation somewhat more efficiently than the 100P or 10P preparations, cell wall quantitation in each rat was derived by referring the counts obtained with a particular tissue to the standard curve made with same cell wall preparation used to inject the rat. The sensitivity of the assays was approximately 10 pg per sample (100 pg/ml). Negative samples were assumed to contain 10 pg per well (i.e., $\log_{10} = 1$) for the purpose of calculations. With the exception of the urines, as measured by the anti-D-ala assay, nearly all tissues from uninjected control rats gave values below background. Sporadic control samples showed values just above background.

RESULTS

Assay specificities. The present understanding of the structure of group A streptococcal cell wall, derived from the studies of Karakawa et al. (12), Schleifer and Krause (18), and Seidl and Schleifer (21), is shown in Fig. 1. The numbers indicate antigenic epitopes which have been described. Our assays recognized the NADG (GlcNAc, no. 1) moiety in the polysaccharide (anti-A assay), the rhamnose oligosaccharide ([Rh-Rh] *n*, no. 2]) of the polysaccharide (anti-A variant), and the D-ala-D-ala moiety (no. 5) of the peptide side chain of the peptidoglycan (anti-D-ala). Figure 2 demonstrates these specificities. The anti-A assay (Fig. 2A) was inhibited by

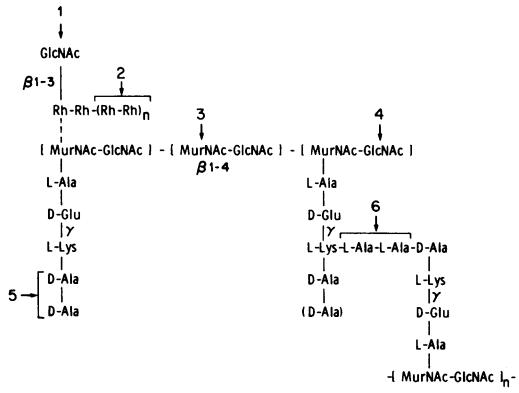

FIG. 1. Current understanding of the structure of streptococcal cell wall. Numbers refer to known antigenic determinants. The radioimmunoassays used in this work recognize the NADG of polysaccharide (epitope 1, GlcNAc); A-variant polysaccharide (polyrhamnose; epitope 2, Rh-Rh); and the D-ala-D-ala end of the pentapeptide side chain of the peptidoglycan (epitope 5). MurNAc, *N*-Acetyl muramic acid.

purified NADG or purified A polysaccharide. A-variant polysaccharide, which is known to lack the terminal NADG groups (3), did not inhibit, nor did monomeric rhamnose or the D-ala-D-ala peptide. The anti-A-variant assay (Fig. 2B) was markedly inhibited by both the A-variant polysaccharide and the A polysaccharide. It was also partially inhibited by NADG, but not by D-ala-D-ala or rhamnose. The anti-D-ala assay (Fig. 2C) was fully inhibited by D-ala-D-ala, but not at all by rhamnose or NADG. It was partially inhibited by A polysaccharide or A-variant polysaccharide, suggesting that these polysaccharide preparations may have been contaminated with peptidoglycan. This possibility was supported by other tests (data not shown) which indicated that the binding of the anti-D-ala antibody to A polysaccharide could be inhibited by the D-ala-D-ala dipeptide and not by NADG.

Distribution of cell wall in rat tissues. The quantity of cell wall material detected by each of the three RIAs in rats injected 4 days previously is tabulated in Table 1. Cell wall material was found in all of the body parts surveyed. In addition, the quantities of cell wall in a given body part as judged by each of the three RIA specificities were roughly comparable and tended to confirm each other (but see below). On day 4, the total amount of cell wall accounted for in all of the body parts assayed amounted to approximately 1 mg compared with a total administered dose of about 10 mg of cell wall per rat.

The initial distribution of cell wall in the front and hind paws, liver, and spleen was statistically compared for each assay and cell preparation by a general linear cell mean model (10). Significantly less 10P material was distributed to the spleen and joints than either 100S or 100P. In addition, more 100S material was generally present than 100P, although these differences were mostly not statistically significant. Finally, the liver or spleen had significantly more cell wall than the front or hind joints, whereas the liver had more than the spleen, and the hind joints had more than the front joints.

To determine whether cell wall material was eliminated by excretion in the urine, 24-h urine specimens were collected over the first 8 days after cell wall injection, and samples were assayed by RIA. The results with the anti-A poly-

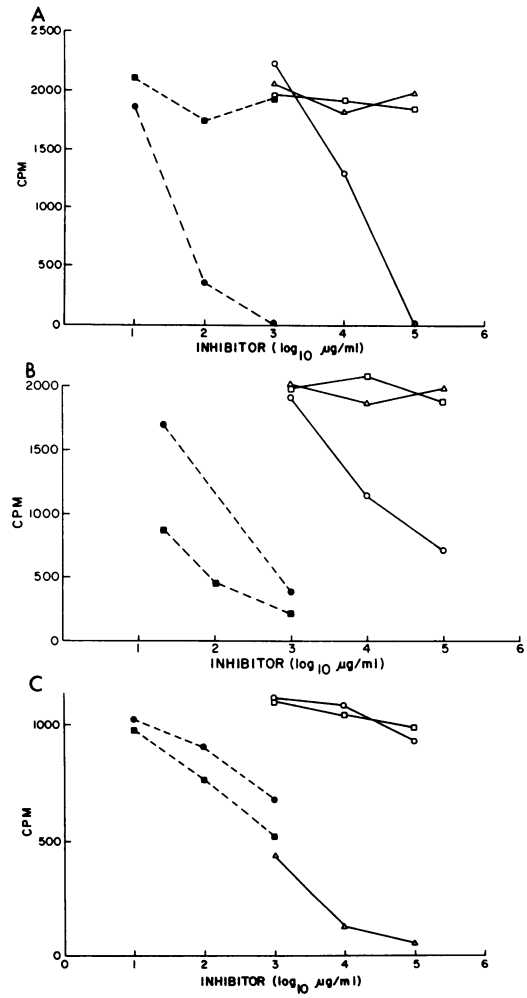

FIG. 2. Inhibition studies with anti-cell wall RIAs. Standard amounts of cell wall were tested in the presence of varying concentrations of inhibitors. Symbols: ●, A polysaccharide; ■, A-variant polysaccharide; △, D-ala-D-ala; □, rhamnose; and ○, *N*-acetyl-D-glucosamine. (A) A polysaccharide RIA; (B) A-variant polysaccharide RIA; and (C) D-ala-D-ala RIA.

TABLE 1. Distribution of streptococcal cell wall in rat tissues on day 4

Body part	100P[a]			100S			10P		
	anti-A	anti-D-ala	anti-A-variant	anti-A	anti-D-ala	anti-A-variant	anti-A	anti-D-ala	anti-A-variant
Liver	8.90[b] ± 0.21	8.15 ± 0.16	7.74 ± 0.19	8.85 ± 0.10	8.88 ± 0.09	8.59 ± 0.03	8.79 ± 0.07	7.96 ± 0.04	7.88 ± 0.07
Spleen	7.95 ± 0.34	8.01 ± 0.25	7.64 ± 0.13	8.41 ± 0.23	8.30 ± 0.26	8.03 ± 0.15	7.71 ± 0.18	8.16 ± 0.20	7.13 ± 0.13
Front paw	6.11 ± 0.13	6.58 ± 0.07	6.24 ± 0.12	6.49 ± 0.02	6.89 ± 0.12	6.41 ± 0.02	4.64 ± 0.61	3.76 ± 0.02	4.85 ± 0.10
Hind paw	6.63 ± 0.26	7.04 ± 0.24	6.60 ± 0.08	6.86 ± 0.11	7.29 ± 0.13	6.62 ± 0.07	5.37 ± 0.13	5.54 ± 1.21	5.05 ± 0.04
Heart	5.91 ± 0.14	5.77 ± 1.42	5.87 ± 0.18	6.07 ± 0.26	6.14 ± 0.15	6.10 ± 0.21	5.48 ± 1.12	4.82 ± 1.44	5.49 ± 0.50
Blood	4.78 ± 0.53	4.40 ± 0.01	4.84 ± 0.14	5.03 ± 0.09	4.40 ± 0.00	5.09 ± 0.29	4.40 ± 0.00	4.40 ± 0.00	5.09 ± 0.13
Lung	6.72 ± 0.55	7.17 ± 0.34	6.46 ± 0.34	6.96 ± 0.21	7.30 ± 0.33	6.60 ± 0.21	6.68 ± 0.43	6.90 ± 0.35	5.98 ± 0.31
Kidney	5.99 ± 0.22	5.77 ± 1.18	6.04 ± 0.27	6.70 ± 0.21	6.58 ± 0.39	6.37 ± 0.17	5.37 ± 0.66	4.53 ± 0.62	5.30 ± 0.19

[a] Four rats were injected with the indicated preparation (30 μg of rhamnose per g of body weight) intraperitoneally on day 0.
[b] Mean $\log_{10}$ picograms of cell wall per organ ± 1 standard deviation. Control rats injected with saline only gave no specific counts per minute in all three RIAs.

FIG. 3. Urinary excretion of streptococcal cell wall as measured by RIA. We collected 24-h urines from injected rats on days 0, 1, 2, 3, 4, and 8. Symbols: ○, 100S; △, 100P; and □, 10P. Urine from control rats was negative.

saccharide assay are shown in Fig. 3. The peak rate of excretion occurred in the first 2 days after cell wall injection and was less than 1 μg per day. Thereafter, excretion gradually decreased until day 8, when the total excreted over 24 h was less than 10% of that seen on day 2. At no time was the amount detected in urine more than a minute fraction of the amount injected. Measurement of urinary cell wall with the anti-A-variant assay gave comparable results (not shown). Interpretation of results with the anti-D-ala assay was difficult because of the high background seen on day 0, representing presumably nonstreptococcal bacterial contamination (data not shown).

Analysis of cell wall material over time. Table 2 shows the results of quantitation of tissue cell wall for the 100S fraction at three time points, using all three antibody assays. Figure 4 depicts similar data obtained with the anti-A assay for all three cell wall preparations. The persistence of cell wall material over time was further analyzed by the method of orthogonal polynomials (17). This statistical approach compares the slope of the plots of cell wall over time, and thereby detects differences in the rates of catabolism as determined by the parameters being compared. The influence of the following variables was assessed: body part (liver, spleen, front paw, hind paw); assay specificity (anti-A, anti-D-ala, and anti-A-variant); and cell wall fraction (10P, 100P, and 100S). Nearly all combinations showed a significant linear decrease of cell wall material with time. The chief exception was the 100P fraction, which showed no significant decrease in the liver by the anti-A-variant and anti-D-ala assays and in the spleen by all three assays

(Fig. 4). The catabolism curves with the different variable combinations were compared statistically. The 100S fraction, as detected by the anti-A assay, decreased more rapidly in the front and hind joints than the 100P fraction. The same trend was seen in the liver and spleen, but statistical analysis could not be made because the slope of the 100P line was horizontal in these tissues. Regarding body parts, the cell wall behaved similarly in the front compared with hind joints and in the liver compared with the spleen. However, the 100S material had a statistically faster rate of decline in the joints compared with the liver and spleen by the D-ala assay. A similar trend was seen with 100P preparation, but statistical comparison again could not be made in most cases. Comparison of the different assays indicated that in the liver and paws the A epitope decreased more rapidly than the A-variant epitope. However, in general, the quantitative results with the three RIAs confirmed one another. Linear regression analysis of the results of individual rat joints calculated separately for days 4, 29, and 65 for all three combinations of the three assays taken two at a time revealed a highly significant ($P < 0.001$) relationship in each case.

Correlation of joint score with cell wall persistence. The course of clinical arthritis in the groups of rats injected with the three cell wall preparations in the current experiments is shown in Fig. 5. The general pattern is similar to what we had previously observed (6), although there are certain differences with these groups of rats and this particular preparation of cell wall fragments (for example in the current experiments, there was more acute arthritis with the 10P and 100P preparations than we had previously seen). For days 4, 29, and 65, linear regression analysis of the joint score of an individual joint versus the quantity of cell wall in that same joint was performed by combining the data from rats injected with the three cell wall preparations. The regression coefficients obtained with each assay at day 4, 29, and 65 are presented in Table 3. In addition, the results obtained with the anti-A polysaccharide assay are depicted in Fig. 6. Joint score and cell wall correlated strongly on day 4. Although a correlation persisted with anti-A and anti-A-variant assays on day 29 and 65, the regression coefficients were progressively lower.

DISCUSSION

We have documented the persistence of streptococcal cell wall material in the joints and other tissues of rats injected with a single arthropathic dose of each of three sizes of cell wall fragments. The RIAs that we used were highly sensitive and specific. We found no problem with nonspecific

TABLE 2. Persistence of 100S fraction of streptococcal cell wall in rat tissues

Body part	Day after cell wall injection								
	4			29			65		
	anti-A	anti-D-ala	anti-A-variant	anti-A	anti-D-ala	anti-A-variant	anti-A	anti-D-ala	anti-A-variant
Liver	8.85[a] ± 0.10	8.88 ± 0.09	8.59 ± 0.03	8.31 ± 0.15	8.66 ± 0.09	8.54 ± 0.06	7.78 ± 0.07	8.23 ± 0.14	8.12 ± 0.10
Spleen	8.41 ± 0.23	8.30 ± 0.26	8.03 ± 0.15	8.19 ± 0.14	8.34 ± 0.18	8.29 ± 0.09	7.67 ± 0.49	7.78 ± 0.27	7.87 ± 0.23
Front paw	6.49 ± 0.02	6.89 ± 0.12	6.41 ± 0.02	5.61 ± 0.21	6.14 ± 0.16	6.11 ± 0.08	4.63 ± 0.38	3.90 ± 0.35	5.35 ± 0.19
Hind paw	6.86 ± 0.11	7.29 ± 0.13	6.62 ± 0.07	6.00 ± 0.16	6.57 ± 0.09	6.22 ± 0.13	5.03 ± 0.16	5.14 ± 0.61	5.72 ± 0.18

[a] Mean $\log_{10}$ picogram of cell wall per body part ± 1 standard deviation.

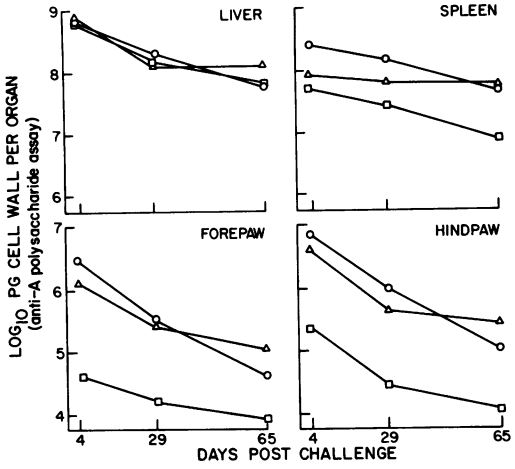

FIG. 4. Persistence of streptococcal cell wall in rats organs. Total organ cell wall was determined by anti-A polysaccharide RIA from rats sacrificed on days 4, 29, and 65. Symbols: ○, 100S; △, 100P; and □, 10P.

factors in tissue homogenates. The high background seen in the assay of urines with the anti-D-ala assay almost certainly indicates specific detection of peptidoglycan associated with bacterial contamination of the urine samples. All bacterial cell walls contain peptidoglycan, and nearly all peptidoglycans have 20 to 70% non-cross-linked pentapeptide side chains terminating in the D-ala-D-ala moiety, which our assay detects. Although part of the terminal D-ala residues are lost in the process of cell wall cross-linking, it would be expected that at least some of these epitopes would be found in every bacterial cell wall. In fact, in other studies (unpublished data), we have found that the anti-D-ala assay indeed detects material from a variety of gram-positive and gram-negative bacteria.

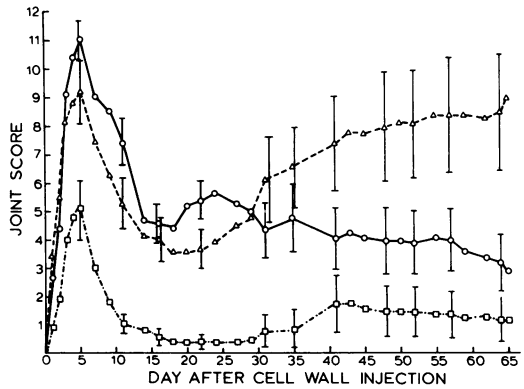

FIG. 5. Mean joint score (total of 4 legs, maximum score is 16) over time after intraperitoneal injection of cell wall preparations representing three size ranges of particles (30 μg of rhamnose per g of body weight). The level of significance (± standard error) among the three groups was calculated for each day that the scores were recorded. For clarity in the graph, the standard error of the mean (bars) was indicated only at 5-day intervals. Fraction 100P (intermediate fragments, △) was significantly different ($P < 0.05$) from fraction 100S (small fragments, ○) at days 59 to 62, and 10P (large fragments, □) is different from 100S at days 1 to 35. There were initially 16 rats per group; 4 rats were killed at day 4, 4 rats were killed at day 29, and the remaining 8 rats were killed at day 65 for measurement of cell wall antigen in tissue.

TABLE 3. Correlation of joint score with cell wall deposition in individual paws

Assay	Day after cell wall injection					
	4		29		65	
	r^a	P	r	P	r	P
anti-A	0.848[a]	<0.001	0.612	<0.01	0.448	<0.01
anti-A-variant	0.839	<0.001	0.667	<0.001	0.387	<0.01
anti-D-ala	0.820	<0.001	0.486	<0.05	0.186	>0.2

[a] Linear regression analysis was by least squares.

The specificity of the anti-A-variant assay deserves some comment. A-variant polysaccharide was used to affinity purify antibody for this assay, whereas the anti-A and anti-D-ala antibodies were purified with synthetic haptens. The A-variant differs from the A polysaccharide by loss of almost all terminal side chain NADG moieties (3). Others have shown that anti-A-variant antisera are inhibited by oligosaccharides derived from this polymer, but not at all by high concentrations of monomeric rhamnose (13, 16), in accord with our results. The partial inhibition of the A-variant assay with NADG may indicate some NADG moieties in the A-variant polysaccharide, either as residual side chain ends or inserted in the polyrhamnose backbone (14). The NADG could cross-react with some other as yet undetermined epitope. It is also probable that the A-variant polysaccharide used to purify the antibody for this assay was contaminated with peptidoglycan, since it partially inhibited the D-ala assay (Fig. 2C). Accordingly, antiglycan backbone reactivities could have been copurified with the anti-A-variant antibody, and these might recognize NADG.

Application of our assays to rat cell wall-induced arthritis has elucidated several of the quantitative aspects of this model. After intraperitoneal injection, the streptococcal cell wall material was disseminated to a variety of organs, but mostly to the liver and the spleen. The cell wall was slowly metabolized so that it persisted throughout the course of the experiment, partic-

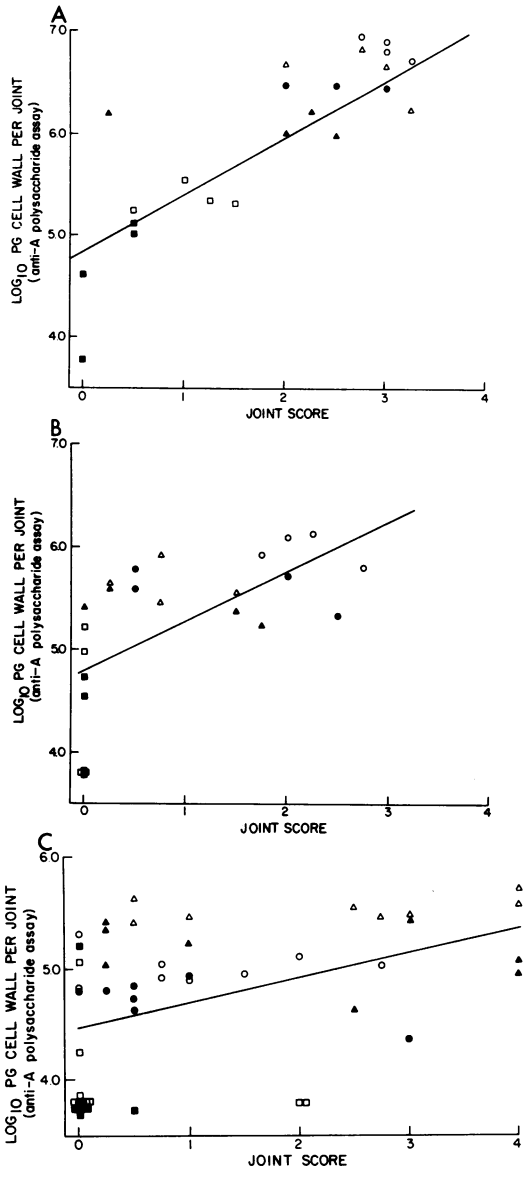

FIG. 6. Relationship of degree of arthritis to quantity of cell wall in individual joints. The clinical joint score is plotted against the total joint cell wall as determined by the anti-A polysaccharide RIA. Open symbols represent hind paws, and closed symbols represent front paws. Symbols: ○ and ●, 100S; △ and ▲, 100P; and □ and ■, 10P. (A) Day 4 after cell wall injection, (B) day 29, and (C) day 65. Lines shown are fitted by least-squares analysis. Correlation coefficients are given in Table 3.

ularly in the liver and spleen. The general distribution of the three cell wall fractions used reflected the course of joint disease with these fractions. Initially, somewhat larger amounts of 100S (smallest fragments) than 100P (intermediate fragments) were deposited in the joints, in parallel with the greater acute disease seen with the former preparation (Fig. 4 and 5). Over time, however, the 100P preparation was more slowly metabolized, reflecting the increased chronic arthritis seen with these larger particles. The 10P preparation (largest fragments) was distributed in substantially lower quantities to the joints, in accord with the much smaller amount of arthritis seen in this case. Additionally, the increased metabolism of the A (NADG) epitope in the liver and paws of 100S-injected rats, compared with the A-variant epitope, suggests that a β-glucosaminidase may slowly remove the terminal NADG, as has been previously reported in rabbit and mouse tissue (19, 20).

We have not yet accounted for the bulk of the injected cell wall material. About 10% of it is found in the organs that we surveyed at 4 days after injection. It is possible that a great deal of it is catabolized in the first 3 days after injection. However, as the amount detected in the urine during this time was less than 1 μg per day, the catabolism must either involve excretion by another route (e.g., feces) or breakdown to fragments too small to be detected in our assays. It is likewise possible that much of the material persists in body regions that we have not yet tested, such as the lymph nodes, the peritoneal cavity, or skin.

An important finding was that the degree of clinical arthritis in an individual joint correlated with the amount of cell wall material deposited in that joint. This correlation was most strong on day 4 and became progressively weaker on days 29 and 65. These results are consistent with the hypothesis that the cell wall material is directly toxic to the joints, and that therefore more cell wall means more inflammation. Since other factors, such as complement, undoubtedly play a role in the chronic disease, it is not surprising that the degree of correlation of cell wall concentration with inflammation decreases with time. There is one caveat to these interpretations. Our correlations were done by pooling data from each of the three cell wall preparations. On day 4, the 100S preparation localized most efficiently to the joints and induced the most arthritis; this might not be a causal relationship. These two findings could be separately related to a third unknown factor specific for that 100S preparation; or they could be associated only by chance. Since there was not enough spread of quantity of cell wall in joints within a group of rats injected with a preparation of one cell wall size, it was impossible to show significant regression without pooling the data.

The restriction of clinical disease in this rat model to the joints can not be explained by a differential localization of cell wall in those

tissues compared with other body parts (Table 1). Indeed, the presence of cell wall in liver and spleen is associated with acute and chronic inflammatory changes (4, 5). The reaction in the liver is characterized by the absence of necrosis, the localization of antigen in the Kupfer cells, and the formation of small granulomata which do not appear to effect hepatic function. The lesions in the spleen and lymph node are characterized initially by focal necrosis and hemorrhage, followed by the accumulation of macrophages containing cell wall antigens. Immune dysfunctions observed in the rats (Schwab, in press) may be related to these histological changes in the lymphoid tissue.

The results presented raise questions that are now approachable with our quantitative technology. What, for example, is the mechanism of distribution of cell wall material after intraperitoneal injection? It could be transported through the blood, although we found very little cell wall in the blood at a given time. It is possible that its distribution depends on low levels of natural antibodies, complement, or both, although other studies indicate that complement is probably not involved in the initial distribution of the cell wall (18a). Another important question is the mechanism of catabolism of the cell wall. Much of it appears to be contained in macrophages based on immunohistological staining (5). It is unknown whether the cell wall is eventually destroyed within the phagocytic vacuoles, egested and broken down extracellularly, or excreted by, for example, the enterohepatic circulation. Ginsburg et al. have addressed this important question of the precise nature of bacterial degradation, and their extensive studies have been recently reviewed (7). It is also not clear whether the material we detect in the tissues is representative of the injected cell wall. Since the cleanest cell wall preparations are still heterogeneous in terms of particle size, it is possible that a special subfraction may be most effective in mediating the tissue inflammation. Finally, we do not yet have any information as to whether the tissue-associated cell wall is bound to any host proteins that might be important in mediating inflammation. The cell wall is known to activate complement (8), and it would not be surprising to find C3 covalently bound to the peptidoglycan components (15). In addition, antibody to the various cell wall epitopes is produced in the arthritic rats (9) and may form immune complexes in vivo. These lines of investigation are currently being pursued with the quantitative methods described here.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AM25733 and AM26574. R.E. is a recipient of an Arthritis Senior Investigator Award from the Arthritis Foundation. A.F. was supported by a postdoctoral research fellowship from the Arthritis Foundation.

Excellent technical assistance was provided by Robert Cheek, Sylvia Craven, Olina McLean, and Roger Brown. Secretarial work was performed by Linda Tillman. Rick Whaley did the statistical manipulations.

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