PERSISTENCE OF GROUP A STREPTOCOCCAL CELL WALLS RELATED TO CHRONIC INFLAMMATION OF RABBIT DERMAL CONNECTIVE TISSUE*

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It has been proposed that the chronic inflammatory process of rheumatic diseases is the direct result of the persistence in tissue of toxic mucopeptide–C polysaccharide complexes from Group A streptococcal cell walls (1–3). In this concept, the tissue damage is produced by the direct toxicity of the mucopeptide moiety. This is supported by the observation that the isolated mucopeptide can induce a severe acute necrotic reaction, and the degree of acute toxicity is inversely related to the amount of residual polysaccharide (2). The polysaccharide, therefore, plays the dual role of protecting the mucopeptide from tissue lysozyme and masking the toxic property. Thus, the cell wall fragments can persist in tissue in a relatively innocuous state, and as the polysaccharide is gradually removed by tissue enzymes the mucopeptide is exposed to produce chronic irritation.

One experimental model of this toxicity is a chronic, relapsing nodular lesion of the dermal connective tissue of rabbits which occurs over a period of at least 92 days following a single injection of a preparation of Group A streptococcal cell walls (3, 4). Chronic inflammatory lesions showing a comparable histologic process also have been produced in rabbit knee joints (1, 5), and mouse hearts (6). The joint lesions were produced by the intra-articular injection of sterile cell wall fragments and the active inflammation was demonstrable for at least 6 wk. Granulomatous lesions involving valves and myocardium were produced by intraperitoneal injection of much larger doses (about 10 mg) of the cell wall fragments.

Heretofore, we have had only indirect evidence that the chronic lesions produced with streptococcal cell walls in these various models are directly related to persistence of cell wall material. This paper describes the relationship of the injected cell wall antigens to the various phases of the relapsing nodular lesion

of rabbit skin. Specific antibodies against both mucopeptide (7) and C polysaccharide have been labeled with fluorescein or ^{125}I, and the fate of the antigens was followed by fluorescence microscopy and radioautography.

The studies described here provide the basis for investigations currently being conducted on the persistence and localization of cell wall material in the experimental models of joint and heart, as well as human material from patients with rheumatoid arthritis or rheumatic heart disease. These experiments are designed to determine as definitively as possible the significance of toxic cell wall fragments in the pathogenesis of connective tissue diseases.

This study also involves the problem of disposal of intact bacterial cells in tissue and the nature of the products of digestion. We are specifically concerned with the question of whether or not potentially toxic cell wall fragments can be released in the natural process of dismantling the cell in vivo.

Materials and Methods

Intact Streptococci.—100 ml of a 16 hr Todd-Hewitt broth culture of Group A streptococci, strain D-58, Type 3 was washed two times with saline at 4°C. The washed packed cells were resuspended in 5-ml sterile pH 6.0 phosphate-buffered saline (0.01 M phosphate, 0.14 M NaCl) and killed by heating at 60°C for 30 min. No viable organisms could be detected by streaking 0.1 ml of the suspension onto sheep blood agar plates. Prior to injection, the suspension was diluted to contain the equivalent of 10 μg of cell walls in 0.2 ml pH 6.0 phosphate-buffered saline (PBS). Higher concentrations of killed bacteria, or the use of viable organisms, produced a necrotic reaction too severe for reliable detection of antigens.

Cell Wall Fragments.—Cell wall fragments from Group A streptococci, strain D-58 were prepared by sonic disruption of cells in a Raytheon 9KC sonic oscillator as previously described (8). The cell wall fragments were treated with 0.026% trypsin at 37°C, washed with buffered saline, and sterilized by graded filtration through 5.0, 3.0, 1.2, and finally 0.45 μ millipore membranes. Cell wall fragments from Group D, strain F-24 streptococci were obtained by treatment of a cell wall preparation for 60 min in the Raytheon sonic oscillator. The cell wall preparation was obtained by disrupting a washed cell suspension in the Braun M S K cell homogenizer. The collection and washing of this cell wall preparation has been described (8). The Group D cell walls were not treated with trypsin because of the autolysis observed with these preparations. Prior to injection, the suspensions were diluted with sterile PBS to contain 240 μg cell walls per 0.2 ml.

Antiserum.—New Zealand white rabbits were immunized intravenously with a Group A Type 3, strain D-58 vaccine according to the procedure of McCarty and Lancefield (9). The cells were grown in trypticase–soy broth (Baltimore Biological Laboratories, Baltimore, Md.), washed three times with saline, and treated with 0.001% trypsin for 4 hr at 37°C. They were then washed two times with saline, resuspended in saline to 20 times the original broth concentration, and held at 62°C for 30 min. The strain used in our studies has no typable M protein but was identified as a Type 3 organism by T protein and recovery of M protein by mouse passage by Mr. W. R. Maxted, Colindale Laboratory, London, England.

Antigens.—Cell walls from Group A streptococci strain D-58 were prepared as previously described (8) except that the cell suspension was disrupted by a 3 min treatment in the Braun M S K cell homogenizer. Mucopeptide (MP) was prepared from ribonuclease- and trypsin-treated cell walls by the hot formamide method of Krause and McCarty (10). The group-specific C polysaccharide (CP) was precipitated from the first formamide extract using acid alcohol and acid acetone according to the procedure of Fuller (11). The mucopep-

tide contained 12% nitrogen and less than 1% rhamnose. Rhamnose and nitrogen were determined by methods previously described (8).

[125]*I Anti-C Polysaccharide (Anti-CP).*—Anti-C polysaccharide was precipitated from the antiserum with the C polysaccharide antigen. The precipitates were washed three times with cold saline and dissociated in 5.0 ml pH 2.75 glycine-HCl-buffered saline for 3 hr at room temperature with constant stirring. Insoluble material was removed by centrifugation at 37,000 g for 60 min at 22°C. The globulin and C polysaccharide were separated on a 2.5 X 33 cm Sephadex G-100 column equilibriated with the glycine buffer. The eluted protein was dialyzed against phosphate-buffered saline, pH 7.2. The final yield was 30% of the initial antibody. This preparation was labeled with ^{125}I as described below.

[125]*I Antimucopeptide (Anti-MP).*—Before any of the sera were absorbed with mucopeptide or applied to tissue sections, they were absorbed with bentonite to remove any lysozyme that might be present. One absorption with 5 mg bentonite per ml serum was carried out at 17°C for 1 hr. Quantitative precipitin tests showed bentonite absorption did not affect the precipitating ability of the antiserum with either MP or CP.

The antimucopeptide was precipitated from the antiserum which had previously been absorbed with C polysaccharide and bentonite. Mucopeptide was added in several steps to a total concentration of 2 mg per ml serum. The washed precipitates were dissociated with glycine buffer as described for anti-CP. The insoluble mucopeptide fragments were separated from the globulin by centrifugation at 37,000 g for 60 min at 22°C. The supernatant was dialyzed against PBS, pH 7.2. The yield was 25-30% and the globulin was 97% specific antimucopeptide. Immunodiffusion and immunoelectrophoretic procedures demonstrated that the specific anti-MP and anti-CP were γG-globulins. That is, they gave lines of identity with DEAE-prepared γG-globulin in Ouchterlony plates, and the immunoelectrophoretic pattern and sedimentation rate were consistent with rabbit γG-globulins.

Normal Globulin.—Pooled normal rabbit serum was absorbed with bentonite and mucopeptide, the latter to remove small concentrations of "normal" antibody. The globulin fraction was twice precipitated with $(NH_4)_2SO_4$ at 50% saturation. The precipitate was dissolved in PBS pH 7.2 and dialyzed against buffer. This crude globulin was labeled with either ^{125}I or fluorescein as described below.

Fluorescein-Labeled Anti-Group A Streptococcus Globulin (FITC anti-A Globulin).—The globulin fraction was twice precipitated from antiserum at 50% saturation with $(NH_4)_2SO_4$. This was conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratories) using the procedure of Marshall, Eveland, and Smith (12). The unconjugated fluorescein was removed by filtration on a Sephadex G-25 column equilibrated with pH 7.2 phosphate buffer.

Fluorescein-Labeled Anti-C Polysaccharide (FITC anti-CP).—The globulin fraction from antiserum absorbed with bentonite and mucopeptide was obtained by $(NH_4)_2SO_4$ precipitation at 50% saturation. This was conjugated with fluorescein as described above.

Fluorescein-Labeled Antimucopeptide (FITC anti-MP).—The globulin fraction from antiserum absorbed with C polysaccharide was obtained by $(NH_4)_2SO_4$ precipitation at 50% saturation. This was conjugated with fluorescein as described above. These globulin preparations were used in the fluorescein technique, instead of purified eluted antibodies, because of the relatively high degree of denaturation of the dilute purified antibody in the fluorescein-labeling procedure.

Iodination of Antibody and Normal Globulin with [125]*I.*—The specific anti-MP, anti-CP, and normal globulin were labeled using a modification of the $NaNO_2$ procedure of Johnson, Day, and Pressman (13). The labeling was carried out to give an average of 1-2 atoms iodine per protein molecule. After removal of unbound iodine by dialysis 2.5 mg/ml bovine plasma albumin and $\frac{1}{10,000}$ merthiolate were added. This mixture was sterilized by H A millipore filtration and stored in the cold. The specific activity of ^{125}I anti-MP, ^{125}I anti-CP, and ^{125}I normal globulin were respectively 30.5 μc/mg, 44 μc/mg, and 54 μc/mg.

Injection of Rabbits and Nodular Lesion Production.—Three groups of New Zealand white rabbits weighing approximately 2.0–2.5 kg were used in the experiments. Group I contained 26 rabbits, group II contained 38 rabbits, and group III contained 39 rabbits. All animals were fed Purina rabbit chow and water *ad libitum.*

Group I: Nine rabbits served as controls, seven of these were injected intradermally with 0.2 ml sterile PBS pH 6.0, and two served as normal uninjected controls. The remaining 16 were injected intradermally with 0.2 ml suspension of intact heat-killed Group A streptococci in sterile PBS pH 6.0. This group of rabbits was examined for the presence of streptococcal antigens using FITC anti-A globulin and FITC normal globulin.

Group II: 4 of the 38 rabbits served as controls, 3 were injected with pH 6.0 buffer, and the 4th was a normal control. The remaining 34 rabbits were injected with streptococci as described above. This group of rabbits was examined for the presence of MP and CP, using FITC anti-MP, anti-CP, and FITC normal globulin as well as the ^{125}I-labeled protein solutions.

Group III: Six rabbits served as controls, five were injected intradermally with 0.2 ml sterile Group D cell wall fragments. The remaining 32 received 0.2 ml of sterile Group A wall fragments. The latter rabbits developed chronic relapsing multinodular lesions. This group was examined using reagents as described for group II.

Collection and Storage of Skin samples.—Rabbits were killed by cervical dislocation and samples obtained by making an incision which cut the site in half; each half was trimmed to about 2 X 5 mm and snap frozen by dipping into liquid propane cooled to −170°C with liquid nitrogen. The frozen samples were stored in screw-cap vials at −70°C until frozen sections were prepared.

In the group I buffer-injected control animals, skin samples were taken at 0, 2, 4, 8, and 12 hr, 1 day, and 2 days after injection. Samples were obtained from the experimental rabbits at 0, 2, 4, 8, and 12 hr; and 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 18 days after injection of the streptococci. One rabbit was taken at each time interval.

In group II, the buffer-injected samples were obtained at 8 hr, 1 day, and 3 days after injection. Samples were obtained from experimental rabbits at 4 hr [1], 8 hr [1], 1 day [1], 3 days [3], 5 days [3], 8 days [2], 10 days [3], 15 days [3], 18 days [3], 21 days [3], 42 days [3], 56 days [3], 84 days [2], 112 days [2], and 168 days [2] after injection. The numbers in brackets represent the number of rabbits taken at each time interval.

Group III represents the rabbits which developed nodular lesions. The control samples, from rabbits injected with Group D cell walls, were taken at 8 hr [2], 1 day [2], and 3 days [1] after injection. Samples from the experimental animals were removed at 4 hr [2], 8 hr [2], 1 day [2], 3 day [2], at the time of the early primary nodular lesion [2], primary nodular lesions [2], subsiding primary nodular lesion [2], early healed primary nodular lesion [2], late healed primary nodular lesion [2], early relapsed (secondary) nodular lesions [2], relapsed nodular lesion [3], subsiding relapsed nodular lesions [3], late healed relapsed nodular lesion [2], no nodular lesion formation at any time up to 54 days [2], primary nodular lesion only and no further gross reaction up to 54 days [1].

Fluorescent Antibody Staining—4-μ frozen sections were prepared and immediately fixed for 30–60 min in absolute methanol. Prior to staining with fluorescein-labeled proteins some of the sections were incubated in 0.1 n HCl for 30 min at room temperature and washed with PBS pH 7.1. This acid treatment greatly decreased the incidence of nonspecific staining of the inflammatory tissue, presumably by removing basic proteins from PMN lysozomes. Nonspecific staining was also controlled by absorbing the FITC conjugates twice with rabbit liver powder according to the procedure of Coons and Kaplan (14).

In the study involving the group I rabbits the reagents used were FITC anti-A globulin and FITC normal globulin. The labeled antibody was diluted to contain 5 μg fluorescein per ml and 0.1 ml was applied to the sections and incubated for 30 min in a humidified chamber

at room temperature. The sections were washed four times with PBS pH 7.1, 10 min each time, and mounted with 90% buffered glycerin. Controls included blocking with homologous anti-A serum, staining with conjugated normal globulin, and sections from buffer-injected and normal uninjected rabbits.

In the rabbits of groups II and III, those sections which were to be stained with the FITC anti-MP reagent were incubated with undiluted, bentonite and MP-absorbed, anti-A serum for 1 hr in a humid chamber at room temperature. After washing 10 min with two changes of PBS, 0.1 ml of the diluted FITC anti-MP reagent was applied and the section treated as described above. When the FITC anti-CP reagent was used, the sections were preincubated with undiluted, bentonite and CP-absorbed, anti-A serum.

Fluorescence microscopy was carried out with a Leitz Ortholux microscope fitted with a HBO200 mercury vapor lamp; heat filters, 4 mm BG38 and 2 mm KG1; exciter filter, 3 mm BG12; Leitz darkfield condenser NA 1.20; 10 X eyepiece fitted with a 2.5 mm OG1 barrier filter.

Radioautography Procedure.—4-μ frozen sections were fixed in absolute methanol, air dried, wetted with PBS, and preincubated 1 hr at room temperature in a humid chamber with undiluted, bentonite and MP-absorbed, normal serum. Following two 5 min washes with PBS approximately 4 μg of the iodinated antibodies or normal globulin contained in 0.1 ml of 50% normal serum (1:1 with pH 7.1 PBS) was applied to the section and incubated 30 min at room tempberature in a humid chamber. The sections were washed four times with PBS, 10 min each, and coated with NTB-2 Nuclear Tract Emulsion (Eastman Kodak Co., Rochester, N. Y.). The coated slides were placed in a Conrad Joftes radioautography chamber and exposed to a low humidity and CO_2 atmosphere for 7–9 days at room temperature.

Controls consisted of blocking with unlabeled specific antibody; use of iodinated normal globulin; and skin sections from buffer-injected, Group D streptococcal cell wall–injected, and normal animals.

Radioautographs were developed at 18 ± 1°C for 4 min with D-19 developer (Kodak), rinsed 15 sec with Kodak SB5 stop bath, fixed for 10 min in acid fixer, and rinsed thoroughly in running water.

Staining Methods.—After examining the fluorescein-labeled sections and recording the location of the areas of interest the cover slips were carefully removed by immersion in absolute methanol and stained with Giemsa stain. After development of the radioautograph slides they were stained through the emulsion with Giemsa in pH 5.75 citrate-phosphate buffer. A tissue section from each skin sample was also stained with hematoxylin and eosin.

Photography.—Fluorescent antibody pictures were taken with a Polaroid MP-3 camera using type 410 (10,000 ASA) film. Brightfield pictures were made using type 42 (200 ASA) film.

RESULTS

Injection of Intact Streptococci and Detection of Antigens with anti-A globulin. —Within the first 4 hr after injection the majority of streptococci were within polymorphonuclear leukocytes (PMN). Neither the intra- or extracellular bacteria were altered significantly in morphology from their characteristic chain of cocci, and fluorescence was clearly associated with identifiable cocci (Figs. 1 *a* and 1 *b*). Labeling of streptococci did not occur after absorption of FITC anti-A globulin with intact streptococci. The greatest change occurred at 24 hr. By this time the total fluorescence in the site had decreased considerably and many of the coccal chains had been broken down to small fluores-

cent particles in PMN. This was the earliest time at which fluorescent material could not be associated with morphologically recognizable streptococci.

By the 2nd-3rd day after injection no streptococci could be identified morphologically. In Giemsa-stained sections most of the cells were mononuclear and contained granular material. This was apparently streptococcal antigen since it corresponded with the pattern of granular fluorescence with FITC anti-A globulin.

The necrotic area increased in size up to day 5 or 6. During this time very large monocytes appeared which contained the cytoplasmic granular material. At about 8 days the size and number of cells began to decrease. At 18-21 days a few mononuclear cells were found to contain small inclusions on staining with Giemsa. Correlating with this histological subsidence, the total fluorescence of the site, as well as the number of cells containing fluorescent material, decreased slowly. From days 5 to 18 the nature of the phagocytized fluorescent material did not change (Figs. 1 c and 1 d).

The buffer-injected control sections displayed a mild inflammatory response which was essentially gone by the 2nd day. These samples contained a few fluorescent cells but this was not associated with streptococcal antigen, as determined by examination of the controls.

Persistence of Mucopeptide and C Polysaccharide following Injection of Intact Streptococci.—In the study involving the group II rabbits both the fluorescent and radioautographic procedures were employed. The FITC anti-MP, FITC anti-CP, and FITC normal globulins were used in the fluorescent procedures.

Fluorescent Antibody Procedure.—4 hr after the injection of intact streptococci, no MP was detected and only a low level of fluorescence was observed with anti-CP reagent. By 8 hr both MP and CP could be detected without difficulty. The characteristic chain of fluorescent rings was seen and most were intracellular. The controls exhibited very few fluorescent cells.

As the necrotic process developed and subsided both MP and CP could be detected. However, between 1 and 3 days after injection the physical nature of the fluorescent labeled material had changed from the characteristic rings to the amorphous granular fluorescence noticed with the FITC anti-A globulin. No MP or CP could be detected after 21 days although the histology of the tissue at 42 and 56 days did not approach normal.

Radioautographic Procedure.—After 4 hr in vivo, MP and CP could be detected both intra- and extracellularly. Partially labeled streptococcal chains were seen with more frequency when stained with the anti-MP antibody than with the anti-CP antibody. In sections labeled with the latter reagent the degree of label was higher. There was an indication in the 8 hr samples that the degree of labeling of MP and CP had increased over that seen at 4 hr. The number of unlabeled streptococcal chains was very low.

At day 1 the concentration of the antigens decreased and continued to de-

crease until 21 days when no MP or CP could be detected. Likewise, in the 42-to 168-day samples, no MP or CP could be detected. Figs. 2 *a–2 f* illustrate the presence of MP and CP at 5 and 18 days and their absence at 21 days.

Nodular Lesion Formation following Injection of Group A Cell Wall Fragments (Group III).—Between 8 and 24 hr after injection the initial edema and erythema reached maximum intensity. This subsided by the 2nd or 3rd day. In 2 of the 33 rabbits no further gross reactions were seen. In the remaining animals firm, red nodules appeared between days 3 and 5, at and around the injection site. Nodular lesions varying in size up to 40 X 40 mm and 6 mm in height were usually reached within 24–48 hr after they first appeared. Some animals developed multinodular lesions containing as many as six nodules. By days 6 and 7 the nodules started to subside and depending on the size of the lesion, completely subsided by day 10 or 12.

Relapsing, (secondary) nodular lesion formation developed between the 14th and 40th day. The nodules usually developed in areas previously involved with the primary nodular lesion. The greatest intensity was reached within 48 hr after first appearing and was generally less than the primary nodules. Complete subsidence varied from 6 to 14 days after the development of the nodules. One animal did not develop a secondary nodular lesion.

Control rabbits injected with Group D cell wall fragments exhibited only erythema and edema which subsided by day 2. Nodular lesions did not develop in these animals.

Histology of Sites Injected with Group A Cell Wall Fragments.—In the period from 2 to 24 hr the connective tissue appeared swollen and contained many PMN's and relatively few mononuclear cells. At day 3 the PMN's had been almost completely replaced by mononuclear cells. Cellular and tissue debris was minimal and collagen bundles appeared undamaged except where the initial edematous process had taken place. Grossly the site appeared normal at this time. As primary nodular lesion developed, the collagen bundles were found to be swollen and fragmented. Many PMN foci surrounded by large and small mononuclear cells were evident during the nodular lesion process (Figs. 3 *a* and 3 *b*). The lesions extended from the epidermis to the deep portion of the dermis adjacent to the panniculus carnosus. Tissue and cellular debris were evident as the lesions subsided.

In the early and late subsided primary nodular lesion samples, taken at 1 and 3 days respectively after the lesions had completely subsided, many fibroblast and large and small mononuclear cells were seen throughout the connective tissue. Most of the mononuclear cells were located in the reticular layer of the dermis. During the development, peak, and subsidence of the secondary nodular lesion the events resembled those of the primary nodular lesion except on a smaller scale.

In those sections obtained from rabbits which displayed no nodular lesion

formation by 54 days, a few collections of mononuclear cells were seen in an otherwise normal skin. The sections from the rabbit which produced only a primary nodular lesion contained small foci of cells throughout the connective tissue. These included fibroblasts as well as large and small mononuclear cells. The rabbit had been grossly negative for 30 days before the sample was taken. Thrombosis of vessels was minimal in all of the sections examined.

Group D Cell Wall Control.—The early inflammatory process paralleled that described in the nodular lesion section above. By the 3rd day, except for the slightly different morphology of the connective tissue at the site, the sections were indistinguishable from normal skin.

Persistence of MP and CP in Sites Injected with Cell Wall Fragments.—Since both the fluorescent and radioautographic procedures gave similar results, only those from the latter will be presented. The acute inflammatory response induced by the Group D cell walls did not exhibit any specific localization of the anti-group A MP– or CP–iodinated globulins (Figs. 3 c–3 f). There was, however, a slightly higher background staining associated with the inflammatory site. This control shows that these reagents fail to react nonspecifically with inflammatory tissue. The control samples taken at day 3 could not be differentiated from the normal uninjected control.

As is illustrated in Figs. 4 a and 4 c, at 4 hr MP and CP could be found both phagocytized and extracellular in the tissue injected with Group A cell walls, the difference in the degree of label was due to the different specific activities of each of the antibodies.

The controls are presented in Figs. 4 b, 4 d, and 4 e. Although the label was not completely blocked by unlabeled antibody, there was a definite decrease in the intensity of silver grains. By day 3, many of the mononuclear cells contained the antigens.

In the primary nodular lesion both antigens were detected. In the late subsided primary nodular lesion, removed about 4 days after the lesion had completely subsided, much of the label was associated with cells in the deep layer of the dermis (Figs. 5 a and 5 b). However, as was true for all samples taken after the maximum primary nodular lesion response, fewer cells contained the antigens. Both antigens could also be detected within macrophages throughout the connective tissue as the secondary nodular lesion developed and reached its maximum (Figs. 5 c and 5 d). Thus, in all the phases of the nodular lesion described so far, both MP and CP could be detected.

In contrast, neither antigen could be detected in either the subsiding (Figs. 5 e and 5 f) or healed scondary nodular lesions. The sample from the rabbit which produced only a primary nodular lesion also was found not to contain MP or CP at 54 days after injection (Figs. 6 e and 6 f). This rabbit had been negative for 30 days before the sample was taken. On the other hand, as is illustrated in Figs. 6 a–6 d, the antigens were detected in the samples from the two rabbits which did not produce the nodular lesions at any time during the

54 days of observation. In all cases where MP and CP were detected they were both found in the same area in consecutive sections.

DISCUSSION

Our understanding of the mechanism by which host tissue degrades bacterial cells is very incomplete. We have even less information on the nature of the products of this degradation and their localization and persistance. If the Group A streptococcus is broken down to small soluble components, it is probably rapidly excreted with no harm to the host, as Schmidt has demonstrated with the isolated group-specific C polysaccharide (15). However, if large complexes of the cell wall are released in the digestive process they may persist in tissue producing profound chronic tissue damage. The process of degradation can be influenced by many factors, including previous contact. Thus, Humphrey and Pagel (16) reported that patients with streptococcal disease tended to degrade heat-killed streptococci in a different manner and rate than normal controls.

We have suggested that the cardiac lesions of rheumatic fever and certain other chronic disorders of connective tissue are due to the localization of bacteria with the subsequent release of toxic cell wall complexes at the site of lesion development. This is compatible with the selective localization and continued tissue injury in the absence of culturable streptococci in human disease (17), and in experimentally infected rabbits (18, 19).

Consistent with this reasoning is the observation in the present study that in rabbits injected with intact streptococci the morphologically identifiable cocci could no longer be detected after 2–3 days; whereas, cell wall antigens were detected 18 days after injection of cells. This suggests that potentially toxic cell wall fragments are released from the streptococcal cell in the process of in vivo degradation.

The results using the specific anti-MP and anti-CP reagents to follow cell wall antigens after injection of intact cells essentially agrees with the observation using the anti-A globulin. The latter reagent, of course, contains antibodies against at least two additional streptococcal antigens, detectable by immunodiffusion. It is not surprising, therefore, that the amount of labeled anti-A globulin reacting with the tissue section decreases steadily over the first 24 hr after injection, whereas, the amount of monospecific anti-MP and anti-CP reagents reacting with tissue sections increases during the first 8 hr of in vivo exposure. This has been confirmed by quantitative in vitro studies in which streptococcal cells exposed to lysozome extracts from rabbit PMN, react with significantly more ^{125}I-labeled anti-MP and anti-CP compared to control cell suspensions in buffer.[1] The reaction of cells with ^{125}I-labeled normal globulin was not affected by this treatment. In other experiments (1) smears of in vitro grown streptococci were shown to react poorly with anti-MP, compared with

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[1] Ohanian, S. H., and J. H. Schwab. Data to be published.

cocci in tissue sections from skin sites injected 4 hr previously. These results are interpreted to mean that, although this strain has no typable M protein, there are antigens superficial to the C polysaccharide as well as to the mucopeptide, which partially mask these cell wall components and are readily removed by tissue factors.

In earlier reports on the experimental model of chronic relapsing lesions in rabbit skin following injection of cell wall fragments, it was postulated that the cell wall material could remain in the tissue and retain its toxic property for a relatively long time (1–3). The present paper demonstrates that the cell wall components of Group A streptococci can persist in the skin tissue of some rabbits for a period of at least 54 days. In more recent studies, in another series of animals observed over a period of 13 wk, relapsing nodular lesions were observed in 3 out of 13 surviving rabbits 92 days after injection of 240 μg of isolated cell wall fragments. Cell wall antigens could be identified in these lesions at 92 days with ^{125}I-labeled anti-MP and anti–group A variant reagents, but antigen having the original group-specific specificity could not be detected.[1]

The persistence of antigen was highly variable between individual rabbits and was correlated with the chronic inflammatory response of the animal, rather than the time elapsed after injection. This is supported by the following observations: (a) Tissue reaction as reflected in the nodular skin lesion was always associated with detectable cell wall antigens. In addition, as judged subjectively from the radioautographs, the amount of antigen present in a tissue section was more related to the phase of the inflammatory lesion than to time after injection. For example, secondary nodular lesions removed from three animals at 15, 27, and 42 days after injection all showed similar histology and comparable density of silver grains in spite of the wide difference in time lapse. (b) In those animals in which the relapsing nodular lesion activity had completely subsided, no antigen was detected. (c) In one animal which displayed only a primary nodular lesion with no further tissue reaction, antigen could not be detected 54 days after injection. These animals eliminated the cell walls in the course of the inflammatory response, and presumably, there would have been no further lesion activity regardless of how much longer they had been observed. (d) In contrast, in those rabbits which showed no nodular lesion reaction at any time, considerable amounts of antigen could still be observed in the area of injection at 54 days. This indicates that the cell wall material can persist in some animals for an indefinite period of time until an adequate inflammatory response is invoked. Presumably, these animals eventually would have developed lesions, since in other studies with this dose of cell wall material an occasional animal would remain completely negative for as long as 8 wk after injection and then develop a typical primary nodular lesion followed by exacerbations (3, 4). The fact that Group A cell wall fragments can reside in tissue within macrophages for a long period before a tissue response is apparent, is one of the most significant observations of this study.

SUMMARY

Antibodies specific for the mucopeptide and group-specific C polysaccharide antigens of Group A streptococcal cell walls were prepared by acid dissociation of immune precipitates, and labeled with either fluorescein or ^{125}I.

Employing both fluorescent and radioautographic procedures the persistence of the antigens was followed in skin sites injected with cell wall fragments. Both antigens persisted within macrophages for at least 54 days in those animals which developed no chronic tissue response. In animals which did develop chronic nodular lesions the concentration of antigen decreased as the inflammatory process subsided. Lesion activity was thus associated with the presence of cell wall material.

The fate of these antigens was also determined following the intradermal injection of intact Group A streptococcal cells. Cell wall antigens persisted in the tissue site considerably longer than morphologically identifiable streptococci, indicating that cell wall fragments are released during dismantling of streptococci in phagocytic cells.

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EXPLANATION OF PLATES

PLATE 109

FIGS. 1 a–1 d. Detection of streptococcal antigens in rabbit skin following intradermal injection of intact Group A streptococci.

FIG. 1 a. 4 hr after injection, stained with fluorescein-labeled anti-A globulin, 25 sec exposure. × 400.

FIG. 1 b. Giemsa stain of Fig. 1 a, showing association of fluorescence with identifiable streptococci. × 500.

FIG. 1 c. 18 days after injection, stained with fluorescein-labeled anti-A globulin, 25 sec exposure. × 400.

FIG. 1 d. Giemsa stain of Fig. 1 c, showing absence of morphologically recognizable streptococci. × 400.
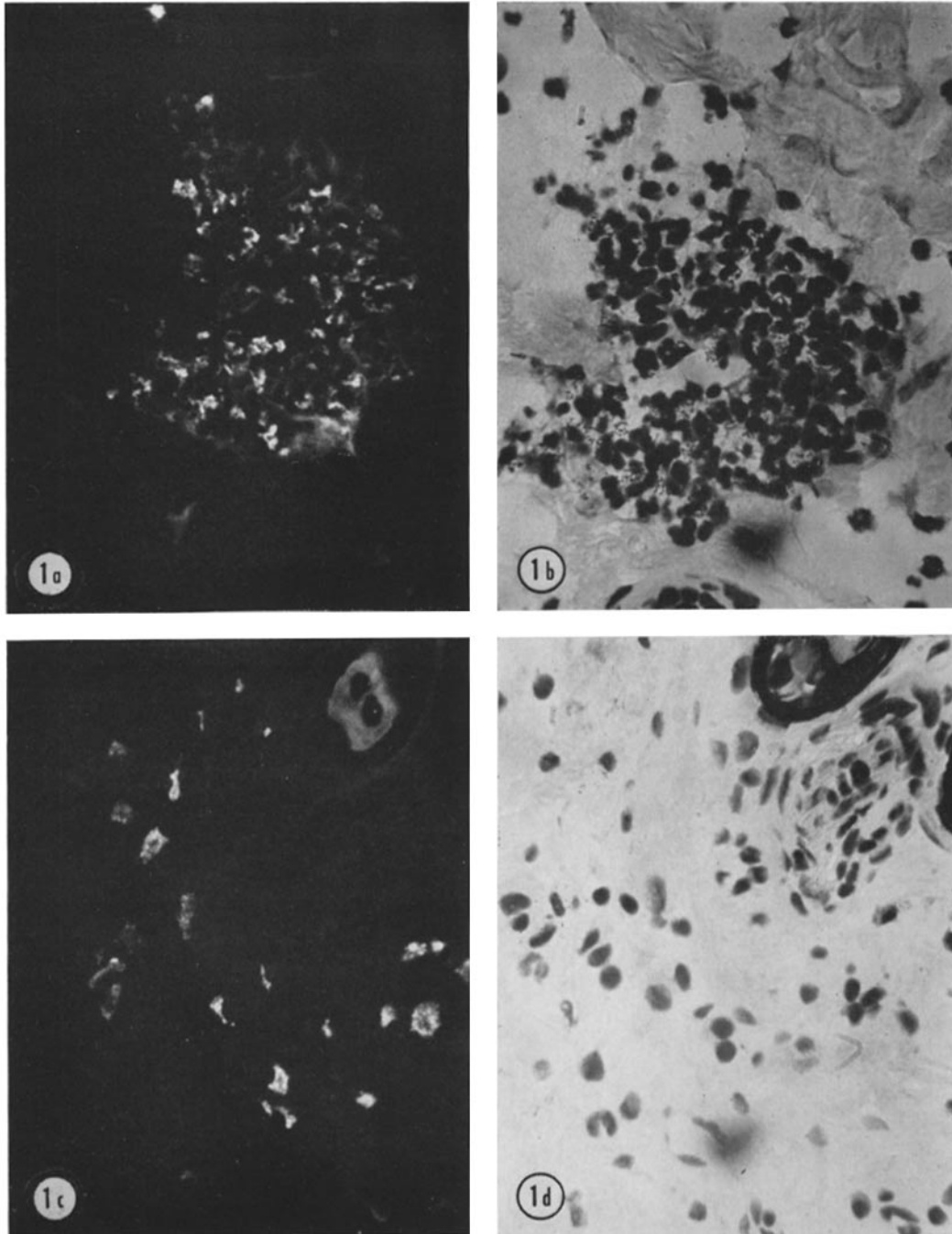
(Ohanian and Schwab: Group A streptococcal cell walls and inflammation)

PLATE 110

FIGS. 2 *a*–2 *f*. Detection of mucopeptide and C polysaccharide antigens in rabbit skin following injection of Group A streptococci.

FIG. 2 *a*. 5 days after injection, stained with ^{125}I anti-MP. $\times$ 845.

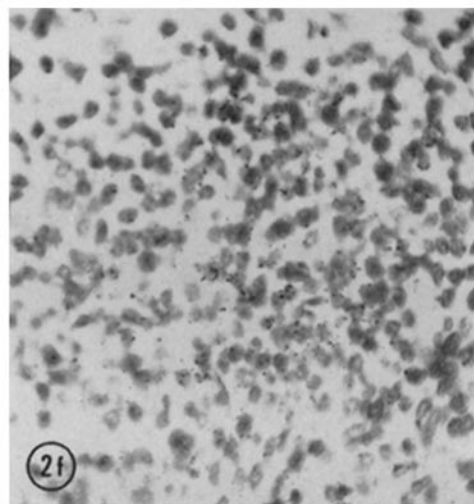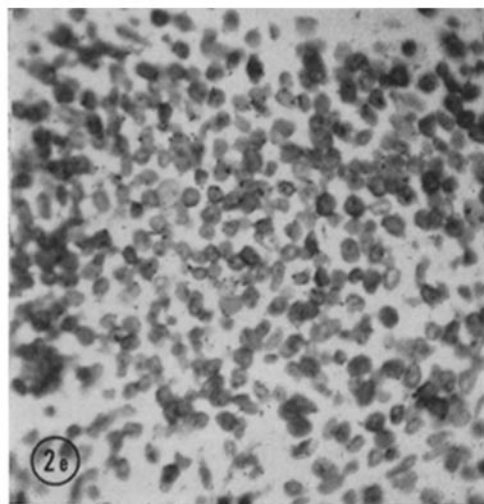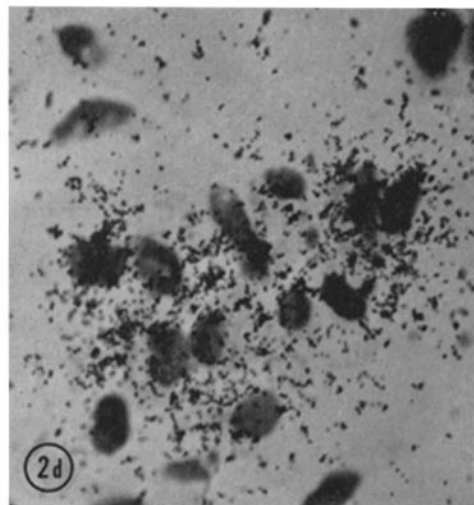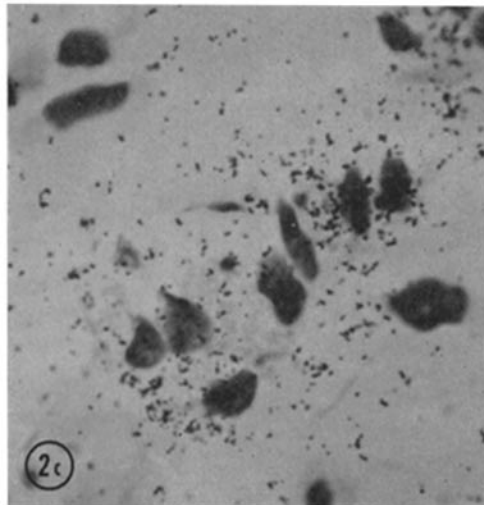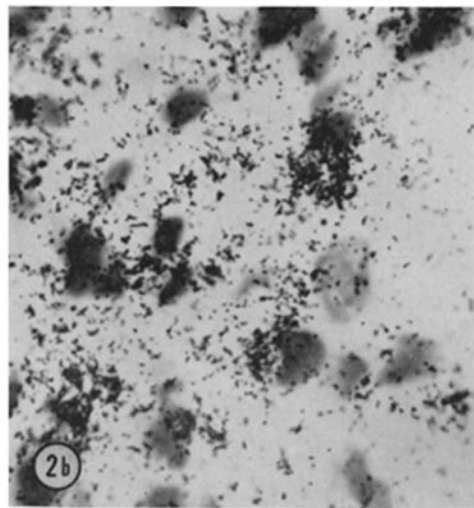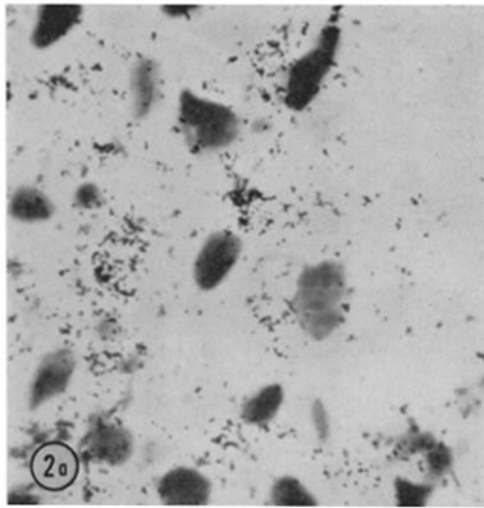FIG. 2 *b*. 5 days after injection, stained with ^{125}I anti-CP. $\times$ 845.

FIG. 2 *c*. 18 days after injection, stained with ^{125}I anti-MP. $\times$ 845.

FIG. 2 *d*. 18 days after injection stained with ^{125}I anti-CP. $\times$ 845.

FIG. 2 *e*. 21 days after injection, stained with ^{125}I anti-MP, showing absence of antigen. $\times$ 375.

FIG. 2 *f*. 21 days after injection, stained with ^{125}I anti-CP. $\times$ 375.

(Ohanian and Schwab: Group A streptococcal cell walls and inflammation)

FIGS. 3 *a*–3 *c*. Primary nodular lesion 4 days after intradermal injection of group A cell wall fragments, showing foci of PMN leukocytes surrounded by mononuclear cells. Giemsa stain.

FIG. 3 *a*. × 88.
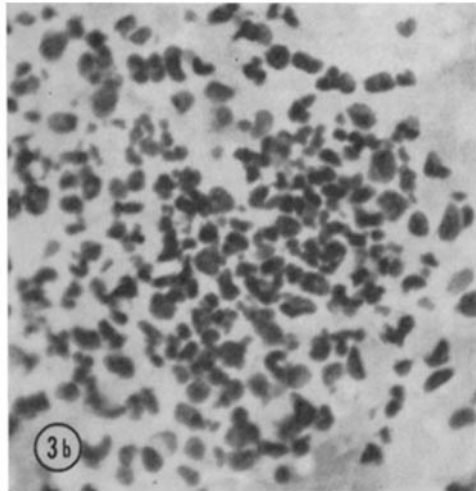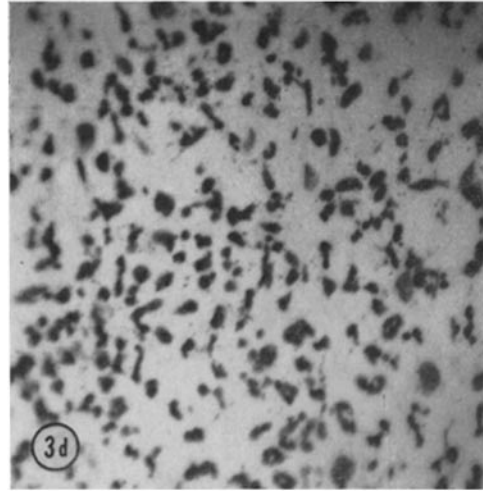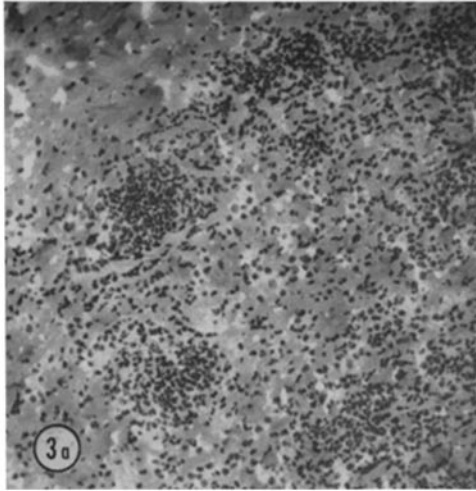
FIG. 3 *b*. × 830.

FIG. 3 *c*, stained with ^{125}I anti-Group A CP. × 365.

FIGS. 3 *d*–3 *f*. Consecutive tissue sections of control sites 8 hr after injection with Group D streptococcal cell wall fragments, showing failure of anti–Group A globulins to react with this control inflammatory tissue.

FIG. 3 *d*. Stained with ^{125}I anti–Group A MP. × 365.

FIG. 3 *e*. Stained with ^{125}I anti–Group A CP. × 365.

FIG. 3 *f*. Stained with ^{125}I normal globulin. × 365.

(Ohanian and Schwab: Group A streptococcal cell walls and inflammation)

FIGS. 4 *a*–4 *e*. Localization of mucopeptide and C polysaccharide 4 hr after injection of Group A cell wall fragments, and typical controls. × 365.
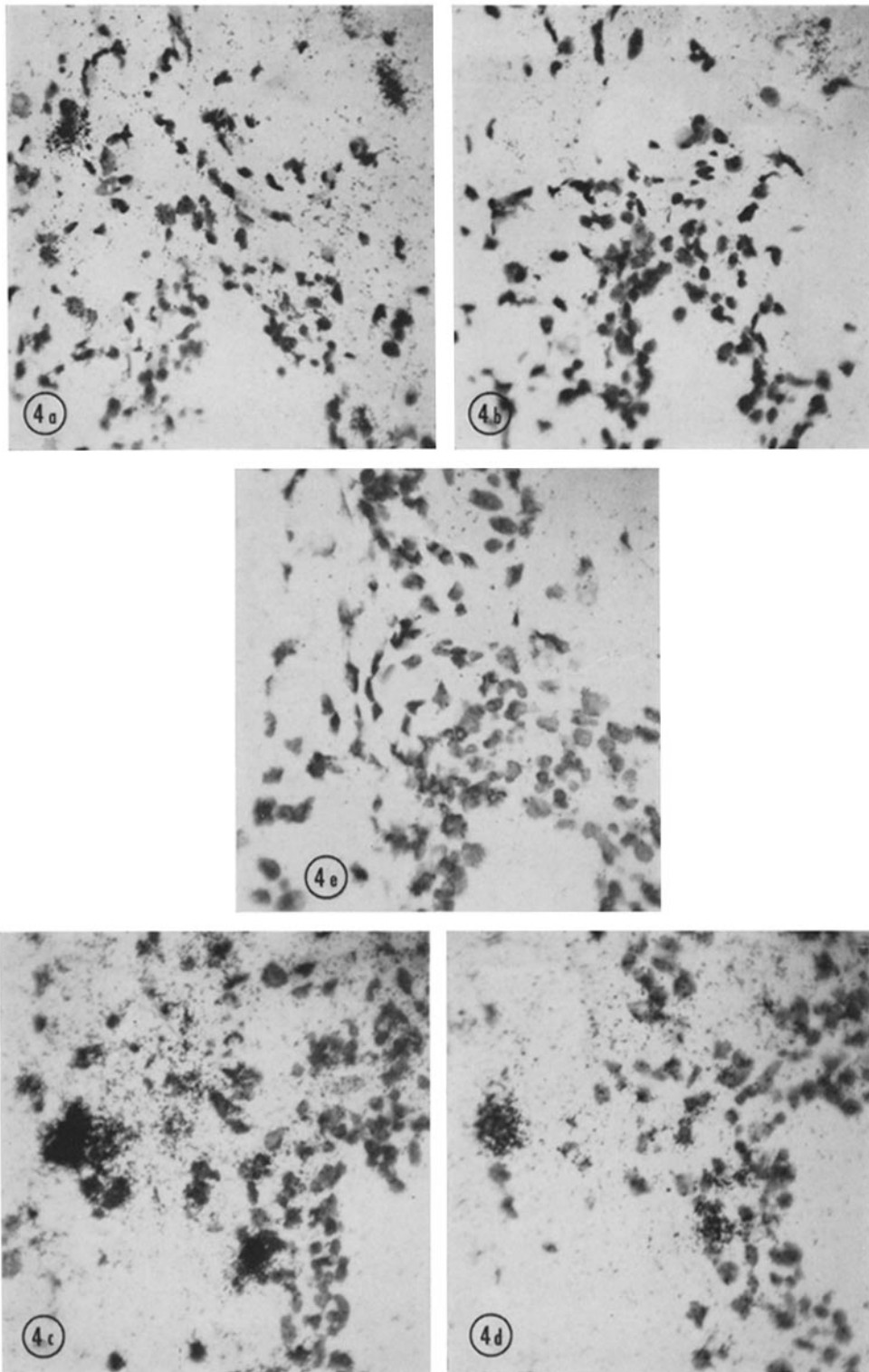
FIG. 4 *a*. Stained with ^{125}I anti-MP.

FIG. 4 *b*. Antibody-blocked control of Fig. 4,*a*.

FIG. 4 *c*. Stained with ^{125}I anti-CP.

FIG. 4 *d*. Antibody-blocked control of Fig. 4 *c*.

FIG. 4 *e*. Stained with ^{125}I normal globulin.

(Ohanian and Schwab: Group A streptococcal cell walls and inflammation)

FIGS. 5 a–5 f. Detection of mucopeptide and C polysaccharide at various stages of nodular lesion development following injection of Group A streptococcal cell wall fragments. × 365.

FIG. 5 a. Late subsided primary nodular lesion 14 days after injection, stained with ^{125}I anti-MP.

FIG. 5 b. Serial section of Fig. 5 a, stained with ^{125}I anti-CP.

FIG. 5 c. Relapsed nodular lesion, 48 hr after appearance, 27 days after injection, stained with anti-MP.

FIG. 5 d. Serial section of Fig. 5 c, stained with anti-CP.

FIG. 5 e. Subsiding relapsed nodular lesion, 30 days after injection, stained with anti-MP, showing absence of antigen.

FIG. 5 f. Serial section of Fig. 5 e, stained with anti-CP.
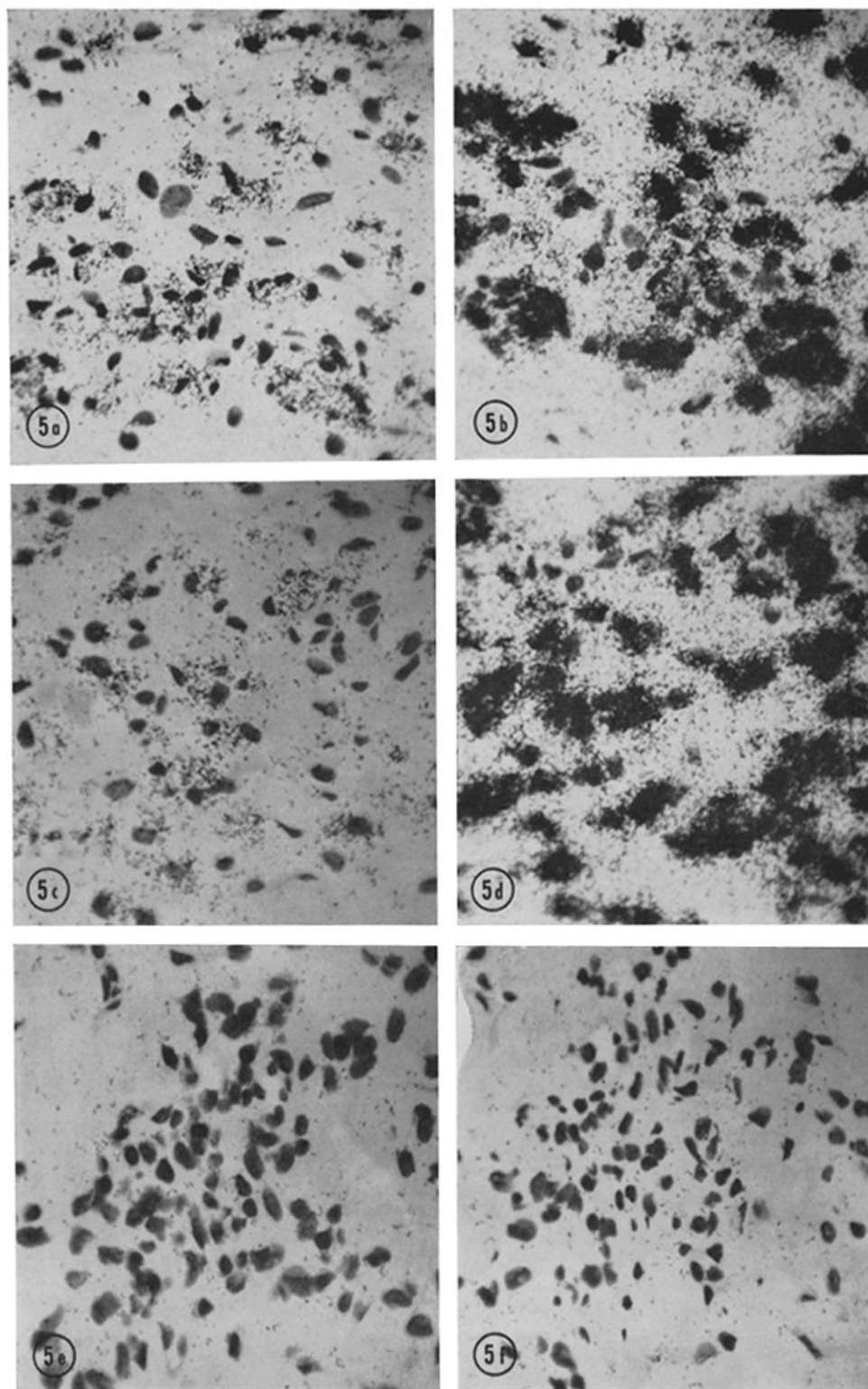
(Ohanian and Schwab: Group A streptococcal cell walls and inflammation)

FIGS. 6 a–6 f. Detection of mucopeptide and C polysaccharide 54 days after injection of Group A streptococcal cell wall fragments. × 390.

FIG. 6 a. Persistence of antigen at injection site in an animal which did not develop nodular lesions during the period of observation. Stained with ^{125}I anti-MP.

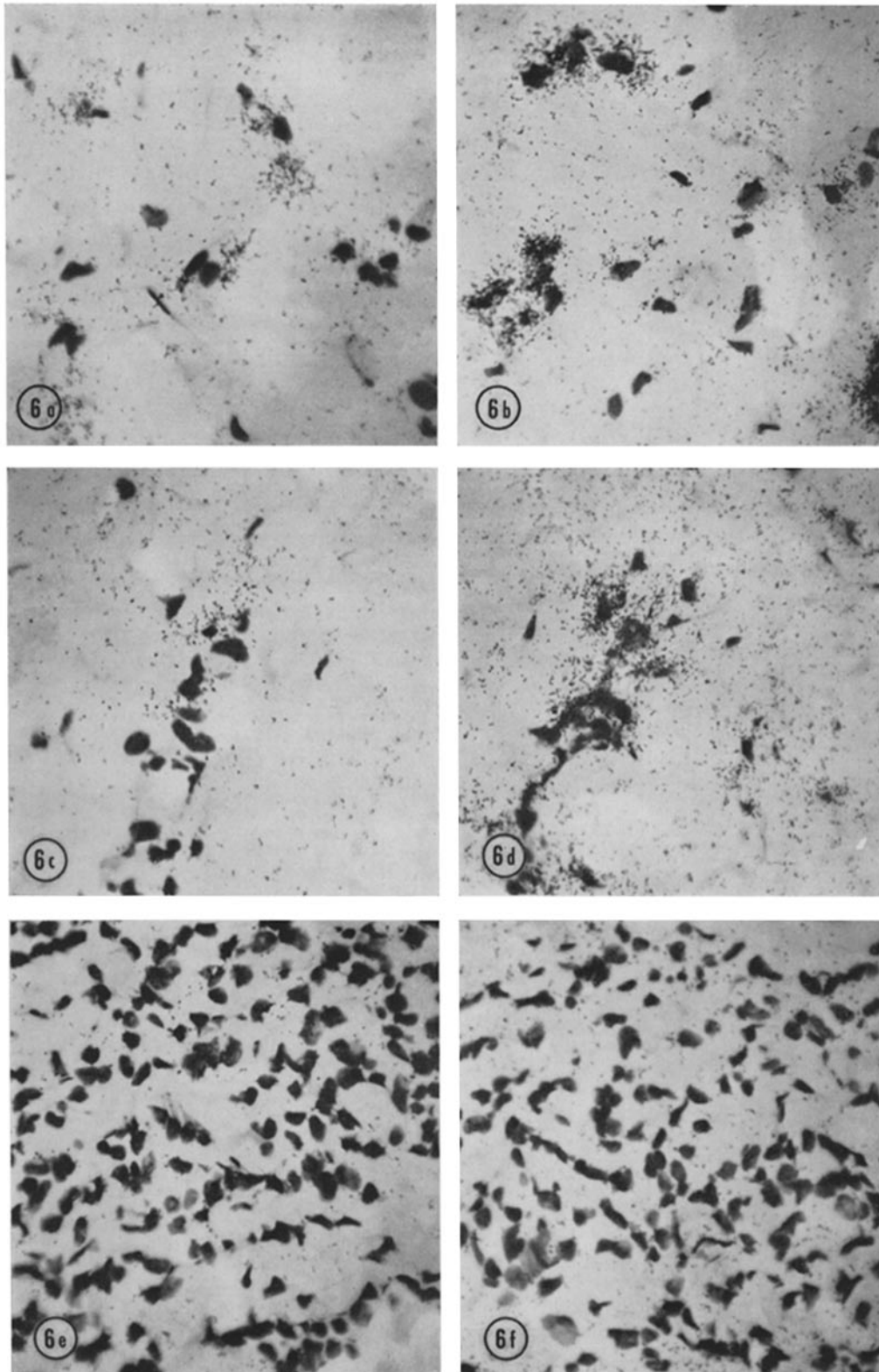FIG. 6 b. Serial section to Fig. 6 a. Stained with ^{125}I anti-CP.

FIG. 6 c. Tissue section from a second rabbit which did not develop nodular lesions over a period of 54 days. Stained with ^{125}I anti-MP.

FIG. 6 d . Serial section to Fig. 6 c. Stained with ^{125}I anti-CP.

FIG. 6 e. Absence of antigen from injection site of a rabbit which had developed only a primary nodular lesion and had been negative for 30 days. Stained with ^{125}I anti-MP.

FIG. 6 f. Serial section to Fig. 6 e. Stained with ^{125}I anti-CP.

(Ohanian and Schwab: Group A streptococcal cell walls and inflammation)