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Induction of Granulomatous Hepatitis in Mice Infected with Group A Streptococci and Treated with Penicillin

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Summary. Mice intravenously infected with group A streptococci and simultaneously treated with penicillin developed a granulomatous hepatitis closely resembling lesions induced by intravenous injection of heat killed streptococci. In these two experimental groups, only the initial phase of granuloma induction differed: in addition to macrophages and lymphocytes, polymorphonuclear granulocytes participated in granulomata elicited by live streptococci. The number and size of liver granulomata in both instances were correlated to the amount of streptococcal material present in the liver as detected by immunofluorescence and electron microscopic techniques. There was no evidence to suggest that penicillin treatment accelerated the elimination of streptococcal components from tissues. The analogy of these experimental liver lesions to "non-specific granulomatous hepatitis" in humans is discussed.

There is relatively little information describing the influence of antibiotic therapy on the intracellular degradation of bacteria and on the elimination of bacterial components from tissues. It has been shown that *in vitro* isolated carbohydrate mucopeptide complexes from the cell wall of group A streptococci, when intravenously injected into mice, elicited a granulomatous hepatitis which lasted for weeks to months (Haferkamp *et al.*, 1970; Heymer *et al.*, 1971). A similar hepatitis arose after the intravenous injection of heat killed group A streptococci into experimental animals (Sellin *et al.*, 1970). In the macrophages of such liver granulomata the carbohydrate mucopeptide structure of the streptococcal cell wall that is responsible for the granulomatous hepatitis, was demonstrable by immunohistological and electron microscopic techniques (Heymer *et al.*, 1971). At the same time a conversion of the group A to A-variant carbohydrate was observed in the macrophages (Heymer and Haferkamp, 1971). In contrast, the intravenous injection of live group A streptococci into mice led to pyemic abscesses in the heart, lungs, kidneys, and liver (Smith *et al.*, 1966a and b). Furthermore, the streptococcal cell wall appeared to be rapidly degraded in and around the abscesses by polymorphonuclear leucocytes. Also, the change of group A to group A-variant carbohydrate was not demonstrable (Schachenmayr *et al.*, 1972).

Our present interest centered on determining whether the use of penicillin in bactericidal doses totally prevented or only modified the development of abscesses in the internal organs of mice intravenously injected with live group A streptococci. In addition observations could be made on whether or not penicillin accelerated the elimination of streptococcal components from tissues, particularly by studying the fate of the most resistant component of streptococci, the cell wall. Finally, an

attempt was made to investigate the basic pathogenic mechanism of the granulomatous tissue response. For this, the histologic reactions observed after the injection of live streptococci and penicillin and after injection of heat killed streptococci were compared with those elicited by injection of biologically inert latex particles.

Materials and Methods

Experimental Animals. Inbred mice of the strain CBA/H (Jackson Laboratories, Bar Harbor, Maine) totaling 186 animals, both sexes, weighing 25–30 grams, were used in these studies. They were fed Altromin R_{1115} (Altromin, Lage, West Germany) and given water ad libidum.

Streptococcal Culture. Group A streptococci, strain T 22/76/2 (type 22) were obtained from the collection of the Rockefeller University, New York, USA. At first, six intraperitoneal (i.p.) mouse passages were employed to increase the virulence of the organisms. Thereafter subcultures were made and were kept at −20° C until used. These subcultures were then thawed, incubated at 37° C in Todd-Hewitt broth (Difco, Detroit, Michigan, USA) and regularly harvested by centrifugation at the end of the exponential growth phase, after 14 hours. The cells were washed twice in saline (4° C) and afterwards suspended in sterile, pyrogen-free saline. The bacterial concentration was ascertained by dry weight (after 18–24 hours at 105° C and 60 minutes in a vacuum desiccator) and rhamnose determination according to Dische and Shettles (1948). The rhamnose content of these group A streptococci at the end of the exponential growth phase was usually about 6–7% of the dry weight of the bacteria. The rapidity of the Dische-Shettles method allowed a standardized injection of streptococci on the same day as they were harvested. All streptococcal suspensions used for infection were proven pure by culture on blood agar.

Streptococcal Infection. One hundred fifteen mice were injected in the dorsal tail vein with 0.5–3.0 mg of live group A streptococci, suspended in 0.1–0.3 ml sterile, pyrogen-free saline. Immediately afterwards each animal received subcutaneously (s.c.) 50000 U penicillin G (Depot-Penicillin Novo: Sodium-Penicillin G and Procaine Penicillin G in the ratio of 1:3; Novo, Mainz, West Germany). The penicillin injections were repeated in the same dosage 16 and 40 hours after infection. Under these conditions 95% of the animals infected with 1.0–2.0 mg streptococci survived the first two days post injection (p.i.) and did not require any other injections of penicillin. Only this group of animals was considered in the evaluation of these experiments. On days 4, 8, 12, 16, 20, 30, 40, 50, 60, 100, and 200 p.i., groups of 4 to 6 mice were sacrificed by heart puncture.

Thirty mice received i.v. 0.1–2.0 mg live group A streptococci suspended in 0.1–0.3 ml sterile pyrogen-free saline without simultaneous or subsequent penicillin injections. Most of these animals died within two to seven days. The surviving animals were bled 8 days p.i.

Control Injection. Twenty mice received only 50000 or 100000 U penicillin G. s.c. given in intervals corresponding to the scheme of penicillin treatment used for the streptococci infected mice. All of these animals were sacrificed 8 days after the last penicillin injection.

Twenty-one mice received i.v. 1.0–3.0 mg of biologically inert latex particles of uniform size: 0.81 μm (Bacto Latex 0.81: Difco, Detroit, Michigan, USA) suspended in 0.2–0.4 ml glycine saline buffer pH 8.2 containing 0.1% sodium lauryl sulfate (Singer *et al.*, 1969). At five minutes, four and 24 hours, 6, 12, and 20 days p.i., three animals of this group were sacrificed.

Examination of Organs from Experimental Animals. Immediately after the bleeding and dissection of the experimental animals, one portion of liver, kidney, spleen, heart, and lung was fixed in 4% formaldehyde solution. A second portion was frozen in liquid nitrogen, and a third part was prepared as described earlier (Heymer *et al.*, 1971) for electron microscopic review. The formaldehyde fixed and paraffin embedded tissues were sectioned and stained with hematoxylin and eosin (HE), and Gram's stain. The paraffin embedding and staining of the tissues of the mice injected with latex particles had to be modified according to the recommendation of Singer *et al.* (1969), because latex particles were eluted from the tissues by xylol or 96% ethanol (Schoenberg *et al.*, 1961). Therefore petroleum ether was substituted for xylol and

isopropanol for ethanol. Thereafter, latex particles could be shown by staining with oil red O (Singer *et al.*, 1969).

Sections of the frozen tissue portions were prepared as earlier described (Heymer *et al.*, 1971). They were examined for group A or A-variant streptococcal C-carbohydrate by immunohistologic staining with fluorescein isothiocyanate (FITC) conjugated A or A-variant antibodies. Two step inhibition studies (Sellin *et al.*, 1970) and further controls generally necessary for the immunofluorescence procedure (Smith *et al.*, 1966) served as criteria for the specificity of the immunofluorescence reaction. The electron microscopic examination of the tissue portions embedded in Araldit (Serva, Heidelberg, West Germany) was carried out as stated previously in detail (Heymer, *et al.*, 1971).

Serological Analysis. The sera of the experimental animals were examined by the capillary precipitin test (Swift *et al.*, 1943) for antibodies against A and A-variant carbohydrates (Heymer and Haferkamp, 1971). To detect antibodies against streptococcal mucopeptides, a modified latex agglutination test was performed (Heymer *et al.*, 1973).

Bacteriological Examinations. The organs and the blood of the experimental animals were tested for the presence of β-hemolytic streptococci by culture of smears or aliquots on blood agar.

Results

Histological Examinations. In the livers of mice infected with group A streptococci and simultaneously treated with penicillin, granulomatous foci appeared 8 to 12 days p.i. Morphologically they corresponded closely to those observed after injection of heat killed group A streptococci (Sellin *et al.*, 1970; Haferkamp *et al.*, 1970). As soon as four days p.i., in the livers of mice injected with living streptococci and penicillin, some aggregations of mononuclear cells were seen. In contrast to mice injected with heat killed streptococci or streptococcal components, they showed in addition to macrophages and lymphocytes, polymorphonuclear leucocytes, and were frequently surrounded by hepatocytes which cytoplasm stained eosinophilically (Fig. 1). Eight days p.i., however, the liver lesions exhibited pure granulomatous character. There were numerous foci composed of large, cytoplasm-rich macrophages surrounded by lymphocytes (Fig. 2). At this time and later, the histological picture of the liver granulomata looked practically the same as observed after injection of killed streptococci. The peak of the granulomatous reaction occurred, as in the earlier examinations, between 12 and 16 days p.i. (Fig. 3). From about the 30th to 40th day p.i., the number and size of the granulomata decreased. As residues of the granulomatous liver lesions, small focal cell infiltrations could be seen by light microscopy until the 200th day of observation. Other than these residues no signs of a chronic inflammatory process in the liver were demonstrable. The extent of granuloma induction was closely related to the quantity of bacteria injected. After injection of less than 1.0 mg of living group A streptococci and penicillin, only sporadic liver granulomata occurred. In contrast, dosages between 1.0 and 2.0 mg produced a marked granulomatous reaction which in some cases showed more than 200 granulomata per section (Figs. 2 and 3).

With Gram's stain, streptococci could be shown in the liver tissue usually until the fourth day, and unfrequently until the eighth day p.i. With the exception of the spleen, the other internal organs of mice infected with streptococci, and simultaneously treated with penicillin, disclosed no evident pathological lesions detectable by light microscopy. The spleen, however, showed a non-specific inflammatory reaction most pronounced between the fourth and twelfth day p.i.

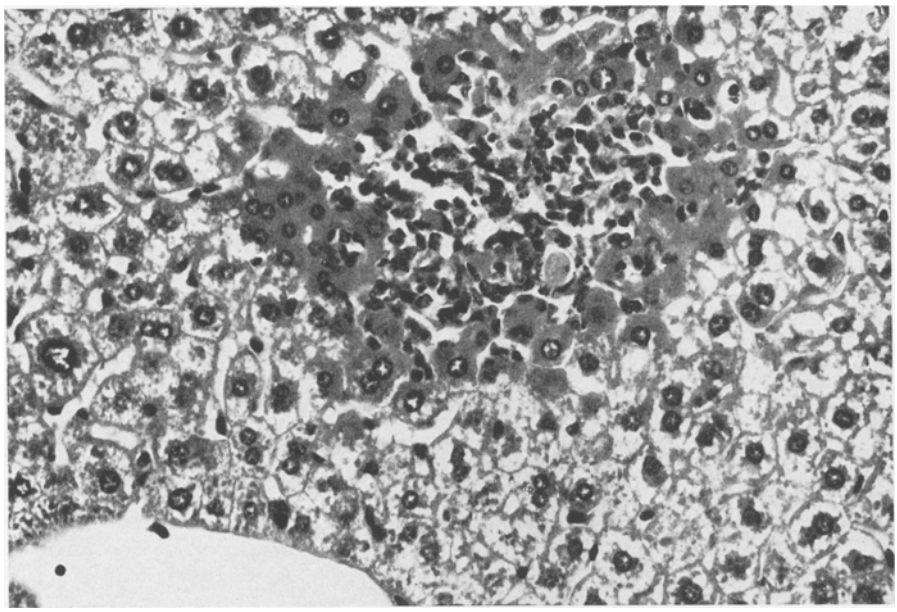


Fig. 1. Liver tissue of a mouse four days after injection of live group A streptococci and penicillin. A granulomatous focus composed of macrophages, lymphocytes, and polymorphonuclear leucocytes is present surrounded by hepatocytes with strongly eosinophilic cytoplasm. HE × 100

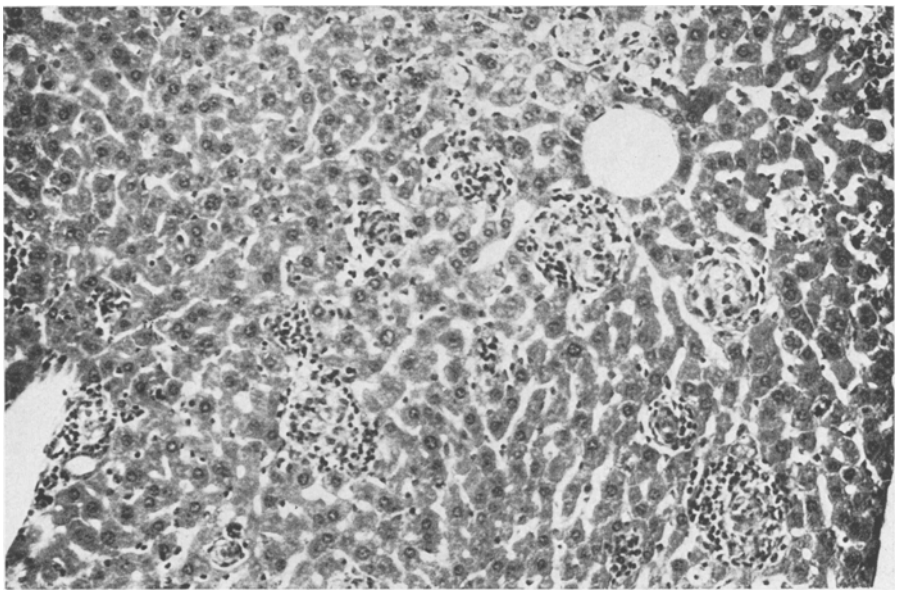


Fig. 2. Liver tissue of a mouse sixteen days after injection of live group A streptococci and penicillin. Numerous liver granulomata consisting of centrally situated macrophages surrounded by a zone of lymphocytes can be seen. No polymorphonuclear leucocytes and no hepatocytes with eosinophilic cytoplasm are visible. HE × 63

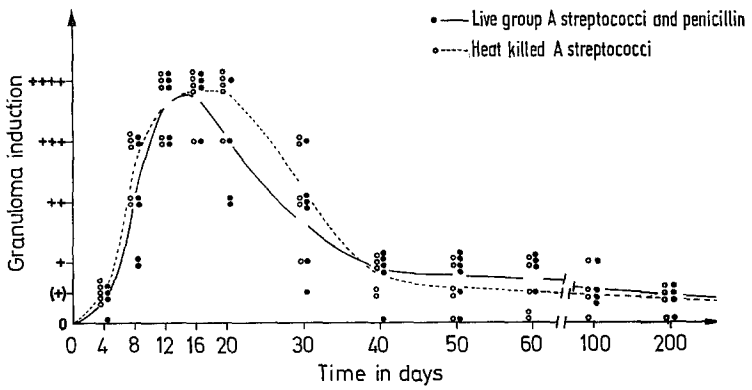


Fig. 3. Development of liver granulomata after the intravenous injection of 1.0–2.0 mg of live group A streptococci and penicillin into CBA/H mice compared to granuloma induction after injection of heat killed group A streptococci. Data illustrating the latter were taken from investigations described previously (Heymer *et al.*, 1971). Each point corresponds to one experimental animal. Semiquantitative evaluation of the presence of granulomata: (+) = single focal collections of mononuclear cells in the liver tissue; + = up to 20; ++ = 20 to 50; +++ = 50 to 150; and ++++ = more than 150 granulomata per section

Immunohistochemical Staining. In cryostat sections of the livers of mice killed between the fourth and 200th day p.i., granular-fluorescent streptococcal antigen deposits could be seen immunohistochemically. The fluorescent material was visible until the fourth day p.i. distributed generally within sinusoids or Kupffer cells. After 8 to 12 days, streptococcal antigens were found only in the areas of the liver granulomata (Fig. 4). The amount of antigen which reacted with the FITC-conjugated A carbohydrate antibodies clearly decreased after the twentieth day p.i. In contrast, after the twentieth day, an increasing amount of deposited streptococcal material reacted with A-variant antibodies. During the following months, a continuous decrease in the total amount of streptococcal antigen could be observed. Nevertheless, a small but constant quantity of antigen was demonstrable immunohistochemically even at the 100th or 200th day p.i. within the granuloma residues.

Electron Microscopical Investigations. Sections of liver tissues of mice infected with group A streptococci and treated with penicillin were studied by electron microscopy through the 200th day p.i. Over the entire period of observation, streptococci or streptococcal components could be detected in the liver. Four days p.i. bacteria were seen within Kupffer cells and rarely within polymorphonuclear leucocytes. At this time foci of mononuclear cells, macrophages and lymphocytes had developed in the liver. In most instances polymorphonuclear leucocytes were infrequently found in these lesions. The marcophages often contained vacuoles filled with streptococci. Between the 12th and 16th day p.i. liver granulomata, which were present in great numbers, almost exclusively contained macrophages and lymphocytes. By contrast, material from the 100th and 200th day p.i. demonstrated only a few small nodules made up of mononuclear cellular elements.

With the electron microscope, streptococci could be shown within the macrophages of liver granulomata between the fourth and 200th day p.i. Whereas

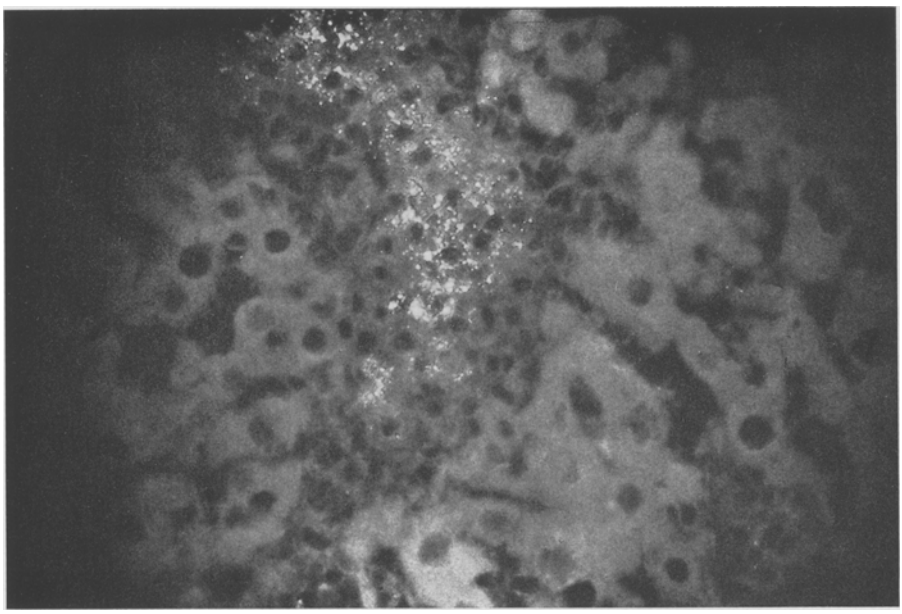
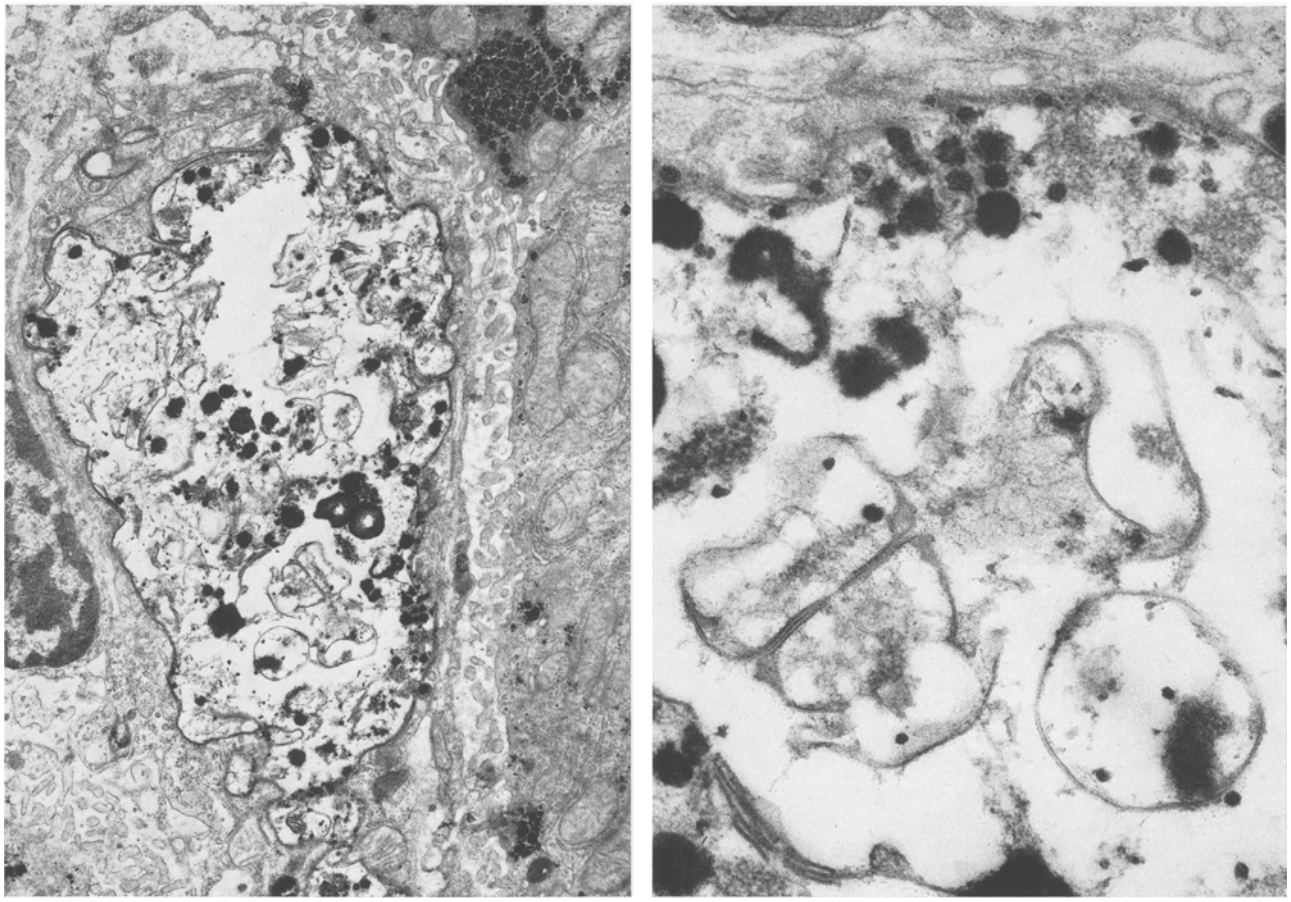


Fig. 4. Immunofluorescence reaction. Mouse liver tissue sixteen days after injection of live group A streptococci and penicillin, treated with FITC-conjugated antibodies against group A carbohydrate. Bright granular fluorescence is present in the area of a granuloma. × 120

macrophages in the liver of mice killed four days p.i. predominantly showed bacteria with intact structures, tissues from animals killed twelve to thirty days p.i. disclosed streptococci with various degenerative alterations. The bacterial cytoplasm showed reticular condensation or was totally destroyed. Most phagocytic vacuoles or phagolysosomes showed streptococcal cell walls with adherent cytoplasmic residues. In these instances the streptococcal cell walls usually exhibited increasing signs of degradation. The amount of streptococcal material present within vacuoles decreased up to the 60th day p.i. However, even at the 100th or 200th day p.i. when only rare mononuclear cell collections were seen in the liver by light microscopy, macrophages were present which contained streptococcal cell walls (Fig. 5).

Streptococcal Infection without Penicillin Protection. Mice which had been injected with 0.1 mg of live group A streptococci and not treated with penicillin, developed four to ten days p.i. abscesses in kidneys and heart, but rarely in liver and lungs. All lesions exhibited a pronounced pyemic character; granulomatous tissue alterations were not seen. The clinical as well as the morphological picture of the streptococcal infection basically corresponded to that seen in earlier investigations (Smith *et al.*, 1966a and b). The injection of more than 0.1 mg live group A streptococci killed the experimental animals within 24–48 hours.

Injection of Penicillin without Streptococcal Infection. Mice which received only three times 50000 U, or three times 100000 U penicillin G s.c. within 48 hours, and were killed eight days after the last injection, showed no liver lesions in histologic sections. The other organs also did not present any pathologic changes.



A B

Fig. 5. Electron micrographs. Mouse liver tissue 100 days after injection of live group A streptococci and penicillin. A. Phagocytic vacuole of a macrophage filled with residues of streptococci. × 14000. B. Higher magnification of this vacuole with distinctly recognizable streptococcal cell wall fragments. × 58400

Table 1. Cultural evidence of β-hemolytic streptococci in organs and blood of experimental animals infected with streptococci: Ø = no growth; ± = single colonies; + = 2 to 10 colonies; ++ = 10 to 20; +++ = 20 to 50; and ++++ = large numbers of colonies on the blood agar plate

Mice Intravenously Injected with Group A Streptococci

Treatment	Without penicillin		With penicillin	
Time of death: (p.i.)	2–24 hours died	8 days sacrificed	2–24 hours died	8 days sacrificed
Liver	++++	+++	+	Ø
Lungs	++++	++++	±	Ø
Spleen	++++	+	+	Ø
Kidneys	++++	++++	Ø	Ø
Blood	+	±	Ø	Ø

Injection of Latex Particles. After the injection of latex particles, no liver alterations were detected with the exception of a slight increase in Kupffer cells. Occasionally, small mononuclear cell infiltrations were found in the neighborhood of the central veins; these were also demonstrable in the livers of some uninjected controls. It could be shown by oil red O staining, as well as by electron microscopy, that latex particles had been phagocytized by Kupffer cells. The histological and ultrastructural picture of latex phagocytosis in the livers was practically identical to corresponding observations of other authors (Singer *et al.*, 1969; Schoenberg *et al.*, 1961; Adlersberg *et al.*, 1969).

Serological Results. Antibodies to A carbohydrate could be demonstrated in only two experimental animals by the capillary precipitin test. In contrast, using a modified latex agglutination test, agglutinating antibodies to streptococcal mucopeptide in titers up to 1:32 were found in sera of all mice infected with streptococci, treated with penicillin, and killed after the 16th day. There was neither a correlation between the appearance of these antibodies and the development of liver granulomata, nor a correlation between the antibody titers and the extent of the granulomatous reaction.

Bacteriological Results. In animals infected with streptococci and not treated with penicillin, pure cultures of β-hemolytic streptococci were usually obtained from various internal organs up to the end of the period of observation. By contrast, group A streptococci could be demonstrated only up to 24 hours p.i. in mice which had been injected with streptococci and treated with penicillin (Table 1).

Discussion

In the described experiments with group A streptococcal infections, mice not protected with penicillin developed pyemic abscesses in various internal organs. In contrast, in the livers of experimental animals infected with streptococci and simultaneously treated with penicillin, granulomata appeared which consisted of macrophages and lymphocytes. By electron microscopy, the persistence of streptococcal cell walls within the macrophages of these liver granulomata was observed. Decrease of granulomatous liver lesions after the 30th day p.i. was correlated with a decrease in cell wall material as shown by immunofluorescence and electron microscopy. Nevertheless, streptococcal antigens could be detected in small

amounts within phagocytes even at 200 days p.i. In older granulomata, group A carbohydrate disappeared and the conversion to group A-variant carbohydrate occurred. This same conversion had previously been found after the intravenous injection of heat killed group A streptococci (Heymer and Haferkamp, 1971). Comparison of these results with those obtained after injection of killed streptococci (Sellin *et al.*, 1970; Haferkamp *et al.*, 1970; Heymer *et al.*, 1971) gave no indication that more rapid degradation and elimination of streptococcal cell walls might result from penicillin treatment.

The relative resistance of the cell wall of certain species of bacteria to enzymatical degradation by macrophages is considered to be a primary cause of granuloma induction (Heymer *et al.*, 1971). However, equivalent amounts of biologically inert latex particles which are not degraded *in vivo* do not initiate granuloma formation.

During the infection of the experimental animals with live streptococci without injection of penicillin, these organisms, possibly due to the leucotactic effect on polymorphonuclear leucocytes, were rapidly phagocytized by such cells, killed, and evidently degraded within a relatively short period of time. Since there was no residue of the cell wall as indicated by negative findings using immunofluorescence and electron microscopic techniques, there was less opportunity for granuloma induction.

The granulomatous reaction of the liver after the i.v. injection of live streptococci and simultaneous dosage of penicillin differed only in the first days p.i. from the tissue alterations seen after i.v. injection of killed streptococci. In the former, granulomata appeared one to two days later, were surrounded by a zone of hepatocytes with eosinophilic cytoplasm and showed the presence of polymorphonuclear leucocytes in this initial phase. In the latter, neither polymorphonuclear leucocytes nor signs of substantial damage to liver cells were apparent. This difference might be explained by the evidence that simultaneously given penicillin did not result in the rapid killing of all streptococci. Isolation of viable streptococci from these tissues within the first 24 hours p.i. supported this premise. Since penicillin inhibits cell wall synthesis of growing bacteria (Strominger and Tipper, 1965; Wise and Park, 1965), streptococci, not dividing, can exert an exotoxin effect on hepatocytes, for example, and can produce leucotactic factors. However, as soon as the streptococci were killed by penicillin, polymorphonuclear leucocytes might no longer be attracted and the killed streptococci would be ingested by macrophages (Kupffer cells). This thereafter induced liver granulomata indistinguishable from those found after injection of heat killed streptococci.

Transposed to human pathology, the described lesions closely resemble those designated "non-specific granulomatous hepatitis" (Terplan, 1971; Eliakim *et al.*, 1968), although the appearance of the latter is based upon different pathogenic mechanisms (Thaler, 1969; Oehlert and Dischler, 1972). However, in the case histories of some patients with such hepatitis, recent infection with pyogenic cocci often treated with antibiotics is described. Therefore, in analogy to the experimental observations presented, a causal connection between pyogenic cocci infections treated with penicillin, and non-specific granulomatous lesions found in human liver biopsies, seems entirely possible.

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