Bacteriological and Pathological Study of Animals Given Freund Adjuvant

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Freund complete adjuvant, with allegedly "killed and dried" Mycobacterium butyricum added (intramuscularly in rabbits and intraperitoneally in mice), produced a local granulomatous lesion and frequently a similar lesion in the viscera. Acid-fast bacilli were present in these lesions, and in some animals M. butyricum was obtained in culture. When this culture was inoculated in mice, a similar lesion developed. Pathological lesions with similar acid-fast organisms also were observed by us in rabbits given Freund complete adjuvant by two other investigators.

Pathological lesions in the viscera have been described in animals injected with Freund adjuvant to which pathogenic and saprophytic mycobacteria, killed tubercle bacilli, and Mycobacterium phlei were added (2, 3, 6, 10). In 1945 Ehrich et al. (1) described the pathological changes in rabbits given an antigen in oil containing alcohol-killed Shigella paradysenteriae. Large numbers of bacteria were demonstrated within the segregating oil drops in the tissues at the site of injection. However, after 14 days the bacteria disappeared. Rupp et al. (9) gave Freund complete adjuvant with M. butyricum added intravenously to rabbits. The reticuloendothelial system was markedly stimulated; however, no acid-fast organisms were demonstrated. M. butyricum added to Freund adjuvant has been given to mice and guinea pigs (4, 8, 9, 11). Rupp et al. (9) cultured the liver of mice, but no organisms were obtained; however, cultures from the guinea pigs were positive in three instances, but the type of organism was not defined.

Freund complete adjuvant with M. butyricum added has been given intraperitoneally to mice to induce amyloidosis (7). A granulomatous lesion has occurred at the site of injection when this adjuvant was given intramuscularly to white Pekin ducks. Acid-fast bacilli were demonstrated in the muscle of each of seven ducks. Fifteen of a group of 20 ducks given this adjuvant intramuscularly developed focal areas of necrosis in the lungs; acid-fast bacilli were demonstrated in these pulmonary lesions.

In this study, rabbits and mice were given Freund adjuvant and were examined bacteriologically and pathologically.

MATERIALS AND METHODS

Freund adjuvant, complete Freund adjuvant, incomplete adjuvant, and killed and dried M. butyricum were obtained from Difco Laboratories, Detroit, Mich. The phosphate-buffered saline solution was prepared by dissolving 0.9 g of Na2O, 0.2 g of KH2PO4, and 8.5 g of sodium chloride in 1,000 ml of distilled water. The pH was adjusted to 7.4. Freund complete adjuvant with M. butyricum was prepared by adding 100 mg of killed and dried M. butyricum organisms to 3.0 ml of the phosphate-buffered saline solution. This suspension of organisms was sonically treated for 5 min in a Branson Sonifer Disruptor. The phosphate-buffered saline suspension of M. butyricum was blended with 3.0 ml of Freund complete adjuvant in a Mulsi Churn; a minimum of 200 strokes was applied. Freund complete and incomplete adjuvants were also prepared by mixing 3.0 ml of each adjuvant with an equal volume of phosphate-buffered saline in the Mulsi Churn, as previously described. The prepared adjuvants were kept at 4.0 C until injected.

Freund complete adjuvant with added M. butyricum (2.0 ml) was given intramuscularly to four rabbits and intraperitoneally (0.5 ml) to 86 mice. The complete adjuvant was given intramuscularly (1.5 ml) to 1 rabbit and intraperitoneally to 40 mice. The incomplete adjuvant (0.4 ml) was given intraperitoneally to 18 mice.

Specimens for histological study were obtained from the viscera and muscle at the site of injection and fixed in a 4.0% solution of formaldehyde. Paraffin sections were prepared and stained routinely.
with hematoxylin and eosin. Sections were stained for acid-fast bacilli by the Fite-Faraco technique. (5).

Tissue for bacterial study was excised by a sterile technique, placed in sterile tubes with 10 ml of sterile saline, and then macerated and shaken on a Vortex mixer. The suspension was diluted to the 10−2 McFarland no. 1 standard. Smears were made and cultures were prepared. The media were Lowenstein-Jensen, Middlebrook, and Albimi Liquid. The Lowenstein-Jensen tubes were incubated at 37 C. The Middlebrook cultures were put into Mylar plastic bags with M. phlei to produce CO₂ and incubated at 37 C. Cultures were observed daily; if no growth was present in 2 weeks, they were discarded.

RESULTS

Experiment 1. Four rabbits were given Freund complete adjuvant with added M. butyricum intramuscularly and killed 28 to 30 days later. Extensive necrosis was present at the site of injection in each rabbit. Acid-fast bacilli, consistent with M. butyricum, were cultured from one rabbit (71-1). Sections of this muscle were positive for acid-fast bacilli. Multiple areas of necrosis, 2 to 8 mm in diameter, were present in the lung of one rabbit and many acid-fast bacilli were present. Tubercles were also present in the liver of two of the four rabbits.

Eight of 86 mice given the complete adjuvant, with added M. butyricum intraperitoneally, died on days 5, 12, 16, 69, and 79. The other 78 mice were killed on days 9, 13, 14, 19, 20, 30, 40, 58, 71, 72, 91, 107, 139, and 140. The abdomen was enlarged in some mice as a result of the acute and chronic reaction in the peritoneum and an enlargement of the liver and spleen. One mouse had ascites. There were white lipid masses, varying in size and number, in the inflammatory reaction in the peritoneal cavity. Polymorphonuclear leukocytes, lymphocytes, and plasma cells infiltrated the fibrous tissue stroma. Nodular deposits frequently were observed on the capsule of the liver. Histologically, these represented local areas of acute and chronic inflammation. Tubercles were present in the liver of many of these mice. Necroses and giant cells were not observed in any of these hepatic tubercles. Similar nodular lesions sometimes were present in the spleen and very often in the lung. A culture was taken from the abdominal tissue of a mouse (71-41) killed on the 71st day and from three mice killed on the 139th day after the intraperitoneal injection; two of these four cultures were positive for acid-fast bacilli, consistent with M. butyricum.

Experiment 2. Freund complete adjuvant was given intraperitoneally (0.25 ml) to 40 mice which were killed 21, 35, 58, 72, 87, and 99 days later. There were white focal areas, representing the adjuvant, between the liver and diaphragm, on the abdominal viscera, and on the peritoneal surface of the abdominal wall. Vacuoles of varying size, polymorphonuclear leukocytes, mononuclear cells, and fibroblasts were present in this inflammatory reaction. No tubercles were found in the viscera, although in some mice nodular lesions were present on the capsule of the liver. Cultures were obtained from the peritoneal cavity of three mice killed on the 21st day, from five killed on the 87th day, and from five killed on the 99th day. These 13 cultures were negative; however, acid-fast bacilli were demonstrable in histological sections from each of these mice.

Experiment 3. Eighteen mice were injected intraperitoneally with 0.4 ml of Freund incomplete adjuvant. One died within 2 hr; five were killed 10 days later, three after 19 days, five after 38 days, and four after 45 days. A gross and histological reaction was present in the abdominal cavity similar to that observed in mice injected with the complete adjuvant, except that the inflammatory reaction was much less in mice given the incomplete adjuvant. No acid-fast organisms were observed in this group of mice.

Experiment 4. Organisms cultured from the necrotic area in the muscle of rabbit 71-1 in experiment 1 were suspended in saline and injected intraperitoneally (0.5 ml) into 24 mice. The mice were killed on the following days: 4, 7, 12, 43, 71, and 100. Microscopically, these mice had a minimal peritoneal reaction when compared to that of the mice given Freund complete adjuvant with added M. butyricum. Microscopically, there was an acute and chronic reaction involving the peritoneum and viscera characterized by polymorphonuclear leukocytes, lymphocytes, and plasma cells. Fibroblasts were conspicuous in some areas. Cultures were taken from two mice killed on day 7, from three killed on day 71, and from two killed on day 100. Two of the seven cultures were positive for M. butyricum, four were negative, and one was contaminated. Acid-fast bacilli were not demonstrated in histological sections from these mice. Fourteen additional mice were given 0.25 ml, intraperitoneally, of this same M. butyricum culture. Acid-fast stains were made on the tissues of four of these mice, and only one was positive for acid-fast organisms.

This same culture of M. butyricum was
given intravenously (1.0 ml) to three rabbits. Two were killed after 11 days and the other after 45 days. No lesions were present in the viscera. Two rabbits were given 0.5 ml of the culture intramuscularly and killed 14 and 18 days later. In the muscle of both rabbits, a local area of necrosis with polymorphonuclear leukocytes, lymphocytes, and mononuclear cells was present. Acid-fast bacilli were present. A culture taken from the abscess in the muscle of one of these two rabbits was negative.

DISCUSSION

Cultures were sterile from mice given Freund complete and incomplete adjuvants intraperitoneally, but cultures were positive for *M. butyricum* from mice given Freund complete adjuvant with added killed and dried *M. butyricum* intraperitoneally and from rabbits given this adjuvant with added organisms intramuscularly. These acid-fast organisms from the rabbit, injected intraperitoneally in mice, produced an acute and chronic peritonitis. Cultures were obtained from seven of these mice; two were positive for butyricum, one was contaminated, and four were sterile. These organisms were given intraperitoneally to 14 mice, 4 of which were examined histologically for acid-fast organism; they were present in the tissues of 1 mouse.

In addition to the demonstration of acid-fast organisms at the site of injection in the mice, acid-fast bacilli were present in the liver of some mice and in the lungs and liver of the rabbit.

The number of organisms in the histological sections of tissue varied. It was not possible in these sections to determine whether these organisms were alive or dead when the tissues were examined. A failure to demonstrate organisms in histological sections may not be interpreted as indicating no infectious process. In our opinion, it was considered probable that some of the *M. butyricum* bacilli “dried and killed” and added to the adjuvant were viable and produced the pathological changes observed in the mice and rabbits.

Multiple injections of Freund complete adjuvant were given intramuscularly to seven rabbits over a period of 289 days by another investigator (Anigstein’s Laboratory) at the Medical Branch. A second investigator (Goldman and Smith’s Laboratory) gave multiple injections of an anti-human colostrum antigen in Freund complete adjuvant into the foot pads of two rabbits. They were killed 102 days after the last injection, and pathological examination was made. Lesions were similar to those observed in the mice and rabbits in our experiment. Acid-fast bacilli were demonstrated at the site of injection in four of the seven rabbits; three of the group were not examined for bacilli. Tubercles were present in the lungs of four rabbits, and acid-fast bacilli were demonstrated in the lung of one. Similar tubercles were present in the liver of two rabbits. No cultures were made from the seven rabbits obtained from the two laboratories. Obviously, we do not know whether these acid-fast bacilli were dead or alive. However, it is of interest to observe that an interval of 102 days occurred between the last injection of the adjuvant and the demonstration of bacilli in the local tissue. It was also of interest to observe bacilli, whether alive or dead, in the lungs and liver of some of these rabbits. Acid-fast bacilli identified as *M. butyricum* were cultured from the rabbit and mouse given Freund complete adjuvant with the addition of dried, dead *M. butyricum* and also were demonstrated in the tissues. Similar histological lesions occurred locally and in the viscera of white Pekin ducks given this complete adjuvant with the added organisms.

The pathological lesions observed in the mice and rabbits given Freund complete adjuvant with added *M. butyricum* were similar to those reported by other investigators (2, 3, 6, 10). The adjuvant used by Jahiel and Koffler (4) was cultured and was sterile. We also were unable to grow any organisms from Freund complete adjuvant or from an ampoule of dried *M. butyricum* organisms.

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