

G-200 columns when either propionic acid or formic acid was used for elution. The phenograms obtained from column-separated polypeptide chains were compared, and no difference was found in either the L chain or H chain banding patterns of γ G-globulin from bursectomized and normal chickens. These results indicate that the subunit of γ G-globulin is the same for bursectomized and normal chickens.

The authors give thanks to Miss Gail Emerson for excellent technical assistance.

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Received March 20, 1967. P.S.E.B.M., 1967, v125.

Production of Anti-*Mycoplasma* (PPLO) Antibodies in Rabbits.* (32140)

HARRY E. MORTON[†] AND RUTH J. ROBERTS

University of Pennsylvania, School of Medicine, Philadelphia, Pa.

Several methods have been used to immunize rabbits with *Mycoplasma* (pleuropneumonia-like organisms, PPLO). These reported procedures ignore quantitation of

*Supported by USPHS Grant AI-04006 from Nat. Inst. of Allergy Infect. Dis.

[†]Present address: Hospital of the University of Pennsylvania, Pepper Laboratory of Clinical Medicine, Philadelphia, Pa.

the protein content of the antigens injected. Frequently the resulting immune sera had relatively low agglutinin titers.

This communication reports experiences with various routes of antigen introduction into rabbits and the results with foot pad inoculation with quantitatively controlled amounts of antigen.

Materials and general methods. New Zealand albino rabbits weighing 4 to 6 lb were used. They were maintained by the Division of Laboratory Animal Medicine. The strains of PPLO were representative of several strains being studied and their sources have been listed previously(1). In every case when the strains were to be used for immunization, the organisms were grown in medium enriched with normal rabbit serum. The procedure for growing the organisms in quantity for antigens has been described in detail(2). Unless mentioned to the contrary, agglutinin titers are expressed as the highest dilution of serum giving definite agglutination by the PPLO-latex agglutination reaction(1). Growth inhibition activity of the sera was determined by the method of Clyde(3). Agar gel diffusion tests were carried out according to the technique of Wadsworth(4). The nitrogen content of the antigens was determined by the usual micro-Kjeldahl method and the value converted to protein content. Freund's incomplete (FIA) and complete adjuvants (FCA) were obtained from Difco Laboratories, Inc., Detroit, Mich. The sodium alginate-calcium gluconate adjuvant (SA-CGA) was obtained from Colab Laboratories, Inc., Chicago Heights, Ill.

The mixtures of saline suspensions of organisms and adjuvants, always in equal proportions, were homogenized by repeatedly drawing into and expelling from a syringe. After disappointing results were obtained from injections into the muscles (IM) of the hips, the shoulders were used because of the reported better lymph drainage from that area. Also when injections were made into the large, central pad of the hind feet, subsequent injections into the shoulders offered greater stimulation of the lymphatic system. Usually 0.5 ml amounts were injected IM in high and low positions in the right and left shoulders. Foot pad (FP) injections of 0.1 ml were made by introducing the needle into the posterior edge of the fleshy part of the central foot pad of each hind leg. Intravenous (IV) injections with saline suspensions of the organisms were made into the marginal vein of the ear. Intracorneal (IC) injections were made under light topical anesthesia.

Experimental procedures and results. Intravenous (IV) route. The classical method of IV injections of saline suspensions of PPLO, strain T-5, on alternate days, as described by Klieneberger-Nobel(5), resulted in sera with relatively low titers. Titers of 1:320 or 1:640 were obtained after a period of immunization extending over 104 days during which the rabbits received the growth from 1,400 ml culture, representing 168 mg protein, in 18 IV injections.

Intracorneal (IC) route. Two rabbits were given their primary antigenic stimulus by injecting IC 0.03 ml saline suspension of *Mycoplasma hominis*, type 1, strain H-34, and on the 28th and 40th days, 0.3 and 1 ml, respectively, were injected IV. Two rabbits on the same dates were injected IM with 2 ml of the mixture of the organisms and SA-CGA. Their two IV injections were 1 ml each. Although sera from all 4 rabbits gave maximum titers of 1:320 to 1:640 by the 48th day, rabbits in the first group received 0.9 mg protein without the benefit of adjuvant and those in the second group received 1.1 mg protein.

Intramuscular (IM) route. Following 11 IM injections in the hips during a period of 13 weeks with Campo-A and 07 strains mixed with FIA, sera with a maximum titer of 1:640 were obtained. Subsequent series of courses of 6 IV injections at 2-day intervals produced sera with titers ranging from 1:640 to 1:10,240 in periods of time ranging up to 6 months. The poor response to the series of IM injections indicated that either the route of administration or the FIA, or both, were unsatisfactory. The subsequent series of IV injections indicated the prior IM injections had served as a primary stimulus, but a weak one, and the rabbits were capable of producing anti-PPLO antibodies. However, about 6 months were required to obtain high titered sera.

Two rabbits received IM in the shoulders a 2 ml mixture of Laidlaw A suspension and SA-CGA which contained the growth from 360 ml culture (14 mg protein). On the 28th day each received 0.1 ml suspension IV. On the 32nd day the serum titers were 1:320 and 1:640. On the 42nd day each received IM

TABLE 1. Typical Antibody Responses in Rabbits Following Injections with Various Strains of *Mycoplasma*: T-5 (Tissue Culture Strain), Laidlaw A (Saprophytic Strain), *Mycoplasma arthritidis* (PG-6), *Mycoplasma hominis*, Type 1 (4330-K) and *M. hominis*, Type 2 (Strains 39 and Campo-W).

| Rabbit no. | 53 | 55 | 54 | 84 | 85 | 86 | 45 | 46 | 82 | 83 | 68 |
|------------|-----------------|---------|---------|-----------|-----------|---------|---------|---------|---------|---------|-------------|
| Day 1 | Titer | T-5 | T-5 | Laidlaw A | Laidlaw A | 1:20 | 39 | 39 | 1:20 | <1:20 | Campo-W |
| | Adjuvant | SA-CGA | SA-CGA | FCA | FCA | PG-6 | SA-CGA | SA-CGA | 4330-K | 4330-K | FCA |
| | Route(s) | FP | FP & IM | FP & IM | FP & IM | FCA | IM | IM | FCA | FCA | FP, IM & ID |
| | Volume | 2 ml | 2.2 ml | 2 ml | 2.2 ml | 2.2 ml | 2 ml | 2 ml | 2 ml | 2.2 ml | 3 ml |
| | Protein | 4.2 mg | 46 mg | 4.2 mg | 46 mg | 22 mg | 3.6 mg | 3.6 mg | 4 mg | 44 mg | 6.6 mg |
| 8 | Titer | 1:640 | 1:2,580 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:320 | 1:320 | 1:160 | 1:640 |
| | Inhibits growth | Yes | Yes | No | No | No | Yes | Yes | Yes | Yes | No |
| 15 | Titer | 1:640 | 1:2,580 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:640 | 1:640 | 1:320 | 1:2,560 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| | Route | IV | IV | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| | Volume | .1 ml | .1 ml | 4.2 mg | 4.2 mg | 4.2 mg | 4.2 mg | 4.2 mg | 4.2 mg | 4.2 mg | 2 ml |
| | Protein | 4.2 mg | 4.2 mg | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 4.4 mg |
| 22 | Titer | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:640 | 1:640 | 1:1,280 | 1:2,560 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| | Route | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | IM |
| | Volume | 4.2 mg | 4.2 mg | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 2 ml |
| | Protein | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 1:2,560 | 4.4 mg |
| 29 | Titer | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| | Route | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| | Volume | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Protein | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | Yes |
| 33 | Route | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| | Volume | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Protein | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | Yes |
| 37 | Titer | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 42 | Titer | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| | Route | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| | Volume | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Protein | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | Yes |
| 44 | Titer | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| 49 | Titer | 1:2,560 | 1:2,560 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:1,280 | 1:20,480 |
| | Inhibits growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |

SA-CGA = sodium alginate-calcium gluconate adjuvant. FCA = Freund's complete adjuvant. FP = injections in foot pads. IM = injections in muscles of shoulders. ID = injections in skin on back. The 4.2 mg protein of strain T-5 represented the growth from 77 ml culture; for strain Laidlaw A, 48 ml culture. The 22 mg protein of strain PG-6 represented the growth from 1,320 ml culture; the 3.6 mg protein of strain 39, 100 ml culture; the 6.6 mg protein of strain Campo-W, 1,600 ml culture and the 4 mg protein of strain 4330-K, 50 ml culture. Titers are the highest dilutions of serum giving a positive reaction in the PPLO-latex agglutination test.

injections similar to the first injections. On the 50th and 57th days 0.1 ml suspension was injected IV into one rabbit and on the 67th day 0.2 ml were injected IV into both rabbits. The rabbits received the growth from a total of 1,102 and 950 ml culture (40 and 35 mg protein), respectively, but the titers on the 70th day were 1:1,280 and 1:320 which are much lower than when the same antigen was administered with FCA by the FP and IM routes (see R#84, Table I).

Three rabbits were each injected IM in the shoulders with 2 ml mixture of saline suspension of Campo-W and SA-CGA. On the 15th day each received IM in the right and left hip 0.9 ml of the same mixture. On the 29th day each rabbit received IV 0.8 ml suspension + 5 μ g endotoxin from *Eberthella typhosa*, 0-901 (kindly supplied by Dr. John M. McKenna, Univ. of Missouri, Columbia, Mo.). When bled on the 38th day the serum titers were 1:640, 1:1,280 and 1:640, respectively. On the 64th day each received IV 1 ml suspension + 5 μ g endotoxin. On the 72nd day the sera had titers of 1:1,280, 1:640 and 1:640, respectively, even though each rabbit had received the growth from a total of 3,700 ml culture. The pooled sera from these rabbits did not inhibit growth of Campo-W. An agar gel diffusion test with a saline extract prepared by sonicating (Model LS-75 sonifier, Bronson Instruments Inc.) a suspension of Campo-W cells with powdered glass (kindly supplied by Mr. Howard Alliger, Heat Systems Co., Great Neck, L. I., N. Y.) for 5 minutes gave 12 lines with the sera. The extract was estimated to contain 12.75 mg protein per ml by the Biuret reaction. The titers of the sera from these rabbits were lower in comparison to the titers of sera from rabbits which received FP, IM and ID injections of the antigen mixed with FCA.

Foot pad (FP) route. The best antibody production, considering the smallest amount of antigen required, the rapidity of antibody production, the higher titers of the sera and the presence of growth-inhibiting properties in the sera, occurred when the primary injection of the antigen was in the foot pad. A single injection of saline suspension of PPLO cells mixed with adjuvant is capable of a satisfac-

tory antibody response (example, R#84, Table I) but a secondary antigenic stimulus by either the IV or IM route enhances production of antibodies. Comparable results were obtained with rabbits immunized with PPLO strain 4330-K.

Simultaneous injections by the FP, IM and ID routes. Since the response to FP injections is rapid and that to IM injections is slower, it appears possible to give the two types of injections, and possibly intradermal (ID) injections as well, at the same time and still have the IM and ID injections serve as delayed booster stimuli. In contrast with the low titers obtained following IM injections of the antigen, even when endotoxin was employed with strain Campo-W, sera of much higher titer were obtained when the strain was mixed with FCA and injected as follows: 0.1 ml in each hind FP, 0.5 ml IM in each of 4 sites in the shoulders and 0.1 ml ID in each of 8 sites on the back (R#68, Table I).

Discussion. PPLO differ from bacteria by not possessing a rigid cell wall and many of the usual enzymes and by possessing a greater lipid content than most bacteria. Their unusual chemical composition may make PPLO poorer antigens than bacteria by failing to stimulate antibody-forming cells or possibly by depressing the activity of the cells. Although the minimum amount of antigen was not determined for the production of agglutinins and growth-inhibiting antibodies, the smallest amount employed was 4.4 mg protein. This is large when one considers that the standard typhoid vaccine contains on the order of 0.5 mg protein per ml. The depressing activity of PPLO on antibody-producing cells is suggested by the work of Copperman and Morton(6) in which mitosis of *in vitro* cultures of lymphocytes initiated by phytohemagglutinin was reversibly suppressed by PPLO.

The IV route appears to be the poorest route for producing antibodies in rabbits against PPLO. Our results are comparable to those obtained by others employing this route and Laidlaw cultures(7), rat strains and the bovine pleuropneumonia organism(8), bovine genital strains(9), a strain from a human knee joint(10), human genital strains and

strains from tissue cultures(11,12,13). The intraperitoneal route is considered to approach the IV route in regard to degree of antibody response. This is borne out with the avian strains(14).

Lemcke(15) made the important observations that, if a series of IV injections was preceded by 2 to 3 weeks with a primary stimulus of the growth from 250 to 500 ml culture mixed with FCA and injected subcutaneously in multiple sites, the complementation titers of the sera were increased 4- to 16-fold and the sera gave better results in the agar gel diffusion tests. If the IV injections were given before the subcutaneous injections, only low titered sera were obtained(16). Although no titers were given, it was stated that satisfactory sera were obtained after 4 months of immunization by a much more complicated method(17) in which the initial IV and IP injections were followed by IM injections using FCA. The technique(18) employing ID injections of the organisms mixed with FCA followed by ID injections of the organisms mixed with FIA and finally an IP injection of the organisms without adjuvant used the growth from only 15 ml culture. No agglutinin titers were given and 4 lines were the maximum number shown in agar gel diffusion tests.

The addition of 5 μ g endotoxin to the antigen was shown markedly to stimulate antibody production in rabbits(19) and for that reason endotoxin was combined with suspensions of PPLO when injected IV in some rabbits. If there was an additional increase in antibody production the increase was less than could be detected by the sensitivity of the test being employed.

Parks *et al*(20) demonstrated that the intracorneal injection of soluble antigen, followed by an IV booster injection of the antigen 27 days later, produced a better antibody response than when the antigen was given IV or IM in comparable doses. Also the response was comparable to, or better than, when the antigen was given IM with FIA. Although the primary IC injection of PPLO cells followed by IV booster injections resulted in sera with titers comparable to those obtained when the primary

injection was by the IM route with adjuvant, neither method appears to produce sera with titers as high as when the primary injection is in the foot pads.

Summary. The antibody response is usually not great when rabbits are injected IV with PPLO or injected IM with the PPLO mixed with adjuvant. The response is better if a primary IM injection of the organisms with adjuvant is followed in a few weeks by one or more IV injections of the organisms. The production of agglutinins is greater following the injection of small amounts of organisms with adjuvant into the hind foot pads and often the response is very good within a period of 7 days. Growth-inhibiting properties have appeared in the sera in a period as short as 14 days following FP injection. Due to the difference in response to FP and IM injections of antigen, injections by both routes may be made at the same time and thus obtain both immediate and prolonged antibody production and sera of higher titer than by either route singly. Intracorneal injection offers a route by which it is possible to obtain a good antigenic stimulus without the use of an adjuvant. Otherwise it appears that the use of an adjuvant is necessary with PPLO for good antibody production. Either Freund's complete adjuvant or the sodium alginate-calcium gluconate adjuvant is satisfactory. Use of the latter adjuvant does not introduce antigens other than those of the *Mycoplasma*.

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Received August 25, 1966. P.S.E.B.M., 1967, v125.

Comparative Effects of Saline and Isoncotic Albumin in Saline on Sodium Excretion.* (32141)

EDWARD KESSLER, RONALD C. HUGHES, CHARLOTTE ORLANDO, AND GHOLAM SHAMLLOU
(Introduced by T. S. Danowski)

*Renal Research Laboratory, St. Elizabeth Hospital, Youngstown, Ohio and Department of
Medicine, University of Pittsburgh, School of Medicine, Pittsburgh, Pa.*

The infusion of isoncotic albumin in saline has been reported to produce the same increase in sodium excretion as saline alone (1,2,3). Although infusion rates were kept constant in relation to animal size by Mills *et al*(1), comparisons were based on different groups of animals. Levinsky and Lalone(2) based their observations not only on different infusion rates relative to the size of the animal, but apparently upon different groups of animals. Isoncotic albumin in saline produced a greater expansion of the vascular volume than saline(1,3), and since Mills and his coworkers(1) could not correlate the increase in blood volume or interstitial volume with the rise in sodium excretion, it seems odd that two solutions with different patterns of extracellular volume expansion should produce the same effect on sodium excretion. The present experiments were undertaken to determine whether isoncotic albumin in saline actually produced the same effect as saline alone. It was reasoned that if the effects were the same, then paired experiments in the same animal performed under comparable conditions should confirm the observations mentioned above(1, 2). The data indicate that under the experi-

mental conditions employed saline infusion produces a greater increase in sodium excretion than does the infusion of isoncotic albumin in saline.

Methods. Twenty-one experiments were performed in 12 female dogs ranging in weight from 7 to 27 kg (mean 14.7 kg). Experiments in 6 dogs were paired and performed at intervals of at least one month with identical infusions of 0.9% NaCl and 6% bovine albumin in 0.9% NaCl infused at a rate of 0.7 or 1.0 ml/kg/min. In half these experiments (dogs 26, 23, 29) saline was infused first and in the remainder (dogs 31, 27, 25) the order of the infusions was reversed. In dog 33 with a similar interval between experiments the saline was infused at 0.6 ml/kg/min, and isoncotic albumin in saline was infused at a rate of 1.0 ml/kg/min. Two single experiments were performed (dogs 28 and 23). Four additional experiments were performed infusing isoncotic albumin in saline at a constant rate until urine flow stabilized for at least 4 periods. The infusion was then suddenly changed to saline and continued at the same rate. In a fifth, the order of infusion was reversed.

The dogs were hydropenic for 24 or 48 hours, and all animals were fasted for 24 hours. Sixteen hours before starting the ex-

*Supported in part by a grant from the Kidney Foundation of Ohio.