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FOWL CHOLERA: CROSS-PROTECTIVE TURKEY ANTISERA AND IgG ANTIBODIES INDUCED WITH *Pasteurella multocida*-INFECTED TISSUE BACTERINS*

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ABSTRACT

Turkey antisera induced with formolized *Pasteurella multocida*-infected tissues (T antisera) passively cross-immunized 48 of 55 chickens against a challenge dose of *P. multocida* organisms, from which 0 of 15 controls survived. However, turkey antisera induced with formalin-killed, agar-cultured *P. multocida* cells (A antisera) passively cross-immunized only 4 of 30 chickens. Cross-immunity refers to protection against a different immunologic type of *P. multocida*. Quantitative precipitin reactions of the A and T antisera with antigens from agar-cultured cells showed that more antibody was present in the A than in the T antisera. However, antigens extracted from the infected tissues reacted with the T and not with the A antisera in the Ouchterlony procedure, demonstrating qualitative differences between the agar-cultured antigens and those extracted from the infected tissue. The gel precipitins isolated from the A and T antisera were characterized as 7S immunoglobulins, which behaved in immunoelectrophoresis as would be expected for a IgG immunoglobulin. The IgG fraction from the T antiserum passively cross-immunized chickens almost as well as the whole antiserum; hence, the IgG antibody is a major factor in cross-immunity.

INTRODUCTION

One of the objectives of our laboratory has been to produce better fowl-cholera bacterins or vaccines, and to this end we have found that formalin-killed, *in vivo*-cultured cells from turkey tissue[1] induced cross-immunity, whereas formalin-killed *in vitro*-cultured cells from agar media induced type-specific immunity only[2]. For example, bacterins[3] prepared with agar-cultured cells (ACB) of *Pasteurella multocida* strain P-1059 (serotype 3) immunized turkeys against challenge with *P. multocida* strain P-1059, but not against strain X-73 (serotype 1). However, liver and heart tissue from a turkey killed by exposure to *P. multocida* strain P-1059 treated with formolized saline solution produced a turkey-tissue bacterin (TTB) that actively cross-immunized turkeys against challenge exposure with *P. multocida* strain X-73.

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

The purposes of the present investigation were: (a) to determine the capability of antisera from cross-immunized turkeys to passively cross-immunize chickens, turkeys, or mice; (b) to determine the class of the antibodies associated with cross-immunity; and (c) to compare the serological reactions of the antibodies in the A antisera with those in the T antisera.

MATERIALS AND METHODS

General. — The cultures, the methods of challenging the immunity, the kinds of chickens, mice, and turkeys, and the conditions under which they were housed and raised have all been previously described[1,2].

Preparation of turkey antisera. — The antisera used in the passive-immunity trials were prepared with three bacterins of *Pasteurella multocida* strain P-1059 (serotype 3). The first bacterin, ACB, consisted of cells grown on agar[2], killed with formalin, and suspended in saline solution at a concentration corresponding to 10 × 1 McFarland Density. The second bacterin, ACB-O, contained the same cell-suspension emulsified with an equal volume of Bayol F mineral oil (Esso Standard Oil Co, Linden, N.J.) containing 3% of Arlacel A* (Atlas Powder, Co, Wilmington, Del.). The third bacterin, TTB, was prepared from the liver and the heart blood of a turkey that had been killed by challenge exposure to strain P-1059. The tissues were homogenized in a Waring blender with formolized saline solution[1]. No viable organisms could be detected in any of the bacterins. All bacterins were administered to turkeys in the same manner: the first inoculation of 1 ml was given subcutaneously (s.c.) in the neck at day 0, the second inoculation of 1 ml was given intramuscularly (i.m.) in the breast on day 35, and the serum was collected on day 49. Antisera induced with ACB or ACB-O bacterins are designated A antisera, and those induced with TTB bacterins as T antisera.

Preparation of rabbit antisera. — Rabbit antisera against whole chicken and turkey serum were prepared by inoculating rabbits with 0.5 ml of the serum emulsified with an equal volume of Freund's complete adjuvant; 0.1 ml was given in the toe pad and 0.9 ml i.m. every week for 4 weeks. The serum was collected from the rabbits after 5 weeks. Rabbit antiserum, monospecific for chicken IgG immunoglobulin was prepared with a purified heavy-chain component of chicken IgG immunoglobulin, as previously described[4].

Passive immunity studies. — The turkey antisera to be tested were given s.c. to 7-day-old chicks or turkey poults, and 24 h later the birds were challenge-inoculated i.m. in the breast, as described in the Tables. The capability of turkey antiserum given intraperitoneally (i.p.) to passively protect mice was evaluated with groups of 20 mice which were inoculated as follows: (a) T-3 antiserum (0.3 ml), (b) T-3 antiserum (0.3 ml) plus fresh normal-serum (0.3 ml), (c) fresh normal serum (0.3 ml), and

*Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U. S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

(d) uninoculated controls. Twenty four hours later, one half of each group was challenge-inoculated with 1,000 *P. multocida* organisms, strain X-73F, serotype 1 given i.p. and the other half of each group was given 1,000 *P. multocida* organisms, strain P-1059, serotype 3.

Storage of antisera. — Antisera were preserved by the addition of phenol and Thimersol to final concentrations of 0.06 and 0.01%, respectively, and stored at 4°. Experiments were performed after 1 month and up to 18 months of storage. Considerable precipitate was formed and this was discarded, but the supernatant remained active when tested in passive-immunity trials or in gel-diffusion reactions.

Antigens. — Type-specific immunogenic endotoxins were isolated by ultracentrifugation from the saline extracts of agar-cultured cells[5]. *P. multocida*-infected turkey livers and heart blood were the source of tissue bacterins[1].

Absorption of antiserum. — Turkey A or T antiserum induced with serotype 3 bacterins was absorbed with nonencapsulated *P. multocida* P-1059B (serotype 3) cells[6] or X-73B (serotype 1) cells as follows: A saline-washed cell suspension (5 ml, 0.75 g of wet cells) was added to the antiserum (5 ml), and the mixture was stirred for 24 h at 4°. The cells were removed by centrifugation, and the supernatant was treated with a similar cell suspension (5 ml). After 3 absorptions, the combined supernatant fluids (total volume ~20 ml) were concentrated to 5 ml by ultrafiltration with a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass. 02173). As a control, a 5-ml sample of the orginal antisera was diluted with phosphate-buffered saline solution (PBS) [0.15M sodium chloride–0.02M phosphate, pH 7.0, containing Na_2HPO_4 (2.5 g), NaH_2PO_4 (0.36 g), and NaCl (8.5 g) per liter] to 20 ml and concentrated back to 5 ml with the same procedure.

Chemical and serologic analyses. — Gel-diffusion precipitin reactions were conducted in a medium containing 1.48M sodium chloride, 0.02M Tris buffer at pH7.2, and 0.8% of agarose at 22°. Immunoelectrophoresis methods have been described[3]. Quantitative precipitin-reactions were conducted in 1.48M sodium chloride with 0.02M Tris buffer at pH 7.2 held at 0° for 2 weeks; the precipitates were centrifuged off at 0° and washed with the same buffer. The amount of precipitated protein was determined by a modified Lowry procedure with crystalline bovine serum albumin as a standard[7].

Preparation of immunoglobulin fraction. — Preliminary purification and concentration of the globulin fraction of the A or T antiserum was as follows: The antiserum (4 ml) was layered over a 50% sucrose solution in 0.02M PBS (0.5 ml), and a 10% sucrose solution in 0.02M PBS (1.0 ml) in a 13 × 50 mm centrifuge tube. The mixture was centrifuged for 17 h at 38,500 r.p.m. at 15° in a SW-39 rotor in a Model L ultracentrifuge (Beckman Instruments, Fullerton, Calif. 92634). Three distinctly colored layers and a gelatinous pellet were produced. The lowest layer (approximately 1.5 ml), just above the pellet, contained most of the serological activity as measured by gel diffusion. Starch-block electrophoresis of the active fraction was conducted[8] in a borate buffer (pH 8.6, 9 g of boric acid and 2 g of sodium hydroxide per liter of

water) at 22° for 17–18 h at a constant current of 15–18 mA and 300 V. Bromophenol Blue was used as a marker. The starch was cut into 1-cm sections and eluted with the buffer (20 ml, pH 6.1, 0.5M sodium chloride and 0.12M phosphate). The absorbancy of the eluate was measured at 280 nm to estimate the protein content. The fractions were concentrated by ultrafiltration to a volume of 2 ml with a PM-30 membrane (Amicon) and were tested for activity by gel diffusion.

RESULTS

Antisera from 4 turkeys induced with serotype 3 *P. multocida*-infected tissues (T-3 antiserum) passively cross-immunized 48 of 55 chicks; antiserum induced with an aqueous suspension of agar-cultured *P. multocida* (A-3 antiserum) cross-immunized only 2 of 15; and antiserum induced with oil-emulsified, agar-cultured cells (AO-3 antiserum) protected only 2 of 15 (Table I, Part A). Titration of the T-3 antiserum from turkey No. 7 and a plot of the data suggests that 0.1 ml of serum would have protected 50% of the chicks. (Table I, Part B).

TABLE I

PASSIVE CROSS-IMMUNIZATION OF 7-DAY-OLD CHICKS WITH *Pasteurella multocida*, SEROTYPE 3, TURKEY ANTISERA AGAINST CHALLENGE WITH SEROTYPE 1

Turkey No.	*Type antisera*	*Amount of serum (ml)*	*Number of survivors/ number of challenged*
Part A			
1	A-3[a]	0.3	2/15
4	AO-3[b]	0.3	2/15
6	T-3[c]	0.3	15/15
7	T-3	0.3	13/15
8	T-3	0.3	13/15
9	T-3	0.3	7/10
Controls			0/15
Part B			
7	T-3	0.15	6/9
7	T-3	0.075	7/19
7	T-3	0.04	0/10

[a]A-3 antisera was induced in turkeys with 2 inoculations of an aqueous suspension of *P. multocida* strain P-1059F serotype 3 agar-cultured cells. [b]AO-3 antisera was induced in turkeys with an Arlacel A Bayol F mineral oil emulsion of the same cells used in A-3. [c]T-3 antisera was induced in turkeys by formolized tissues from a turkey that had been killed with *P. multocida* strain P-1059F serotype 3. All groups were inoculated by the same route and at the same time, and antiserum was collected 7 weeks after the first inoculation.

By use of the same procedure, we found that 0.4 ml of T-3 antiserum passively cross-protected 8 of 11 seven-day-old chicks and 7 of 11 seven-day-old poults; 0.3 ml protected 7 of 11 chicks and 5 of 11 poults; 0 of 22 controls survived the challenge dose of serotype 1 *P. multocida* organisms. Thus, turkey poults are almost as easily immunized as chicks.

No passive type-specific or cross protection was conferred to mice with the same turkey antiserum that passively cross-protected the turkeys, even when fresh, normal turkey serum was added as a source of turkey complement.

Quantitative precipitin determinations of the reaction of A-3, AO-3, and T-3 antisera with the type-specific free endotoxin isolated from agar-cultured serotype 3 *P. multocida* cells are reported in Table II. The AO-3 antiserum gave almost 10 times as much precipitate as any of T-3 antisera. Tests of the supernatants with excess antigen or with excess antisera gave no clear cut equivalence point. Neither the AO-3 nor the T-3 antisera reacted with the endotoxin of the challenge strain, X-73, serotype 1. Obviously, the cross-protective antibody cannot be measured by its reaction with endotoxin.

Quantitative precipitin determinations of the reaction of A-3, AO-3, and T-3 antisera with a mixture of antigens found in the saline extracts of the cells are also reported in Table II for comparison.

TABLE II

QUANTITATIVE PRECIPITIN REACTION OF *Pasteurella multocida*, SEROTYPE 3, TURKEY ANTISERA WITH *P. multocida* TYPES 1 AND 3 ENDOTOXIN, AND WITH THE SALINE EXTRACT FROM *P. multocida cells* OF SEROTYPES 3 AND 1

Antigen added (μg)	*Total amount of protein precipitated at 0° (μg/ml of antisera)*[a]					
	Antisera induced with agar-cultured cells		*Antisera induced with infected tissue*			
	1 A-3	*4 AO-3*	*6 T-3*	*7 T-3*	*8 T-3*	*9 T-3*
Type 3 endotoxin						
102	35	570	65	80	120	
415	40	1300	80	150	120	
1600	130	2000	240	150	190	
Type 1 endotoxin						
100	0	0	0		0	0
1600	0	0	0		0	0
Serotype 3, saline extract 14,700	320	1700		500	425	320
Serotype 1, saline extract 18,300	332		690		564	

[a]The serum numbers refer to those used in Table I.

Absorption of T-3 antiserum with agar-cultured serotypes 1 or 3 *P. multocida* cells effectively removed all of the protective antibody, as shown in Table III.

The serotype 3 cells absorbed all of the gel precipitins, and the serotype 1 cells absorbed part of the precipitins from the AO-3 antiserum (Fig. 1A). Either cell type absorbed all of the precipitins from the T-3 antiserum (Fig. 1B).

An aliquot of the same cells that absorbed the protective antibody from the

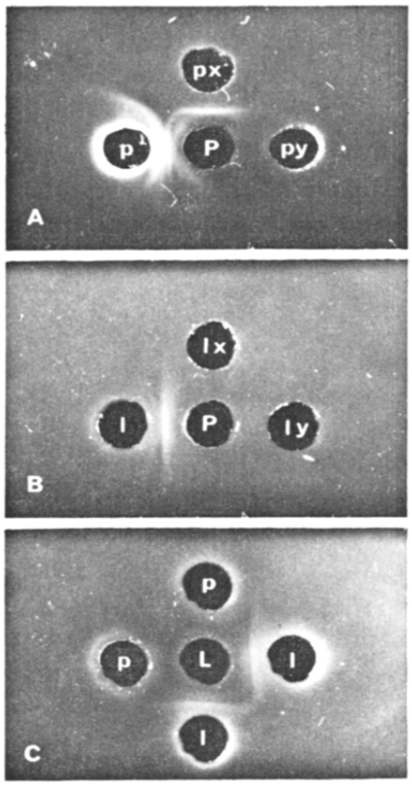

Fig. 1. Gel-diffusion precipitin patterns with *Pasteurella multocida* turkey antisera, and saline-extracted antigens of *P. multocida*. *A:* Center well (P), saline extract of serotype 3 agar-cultured cells; left well (p), antisera to agar-cultured cells; top well (px), antisera absorbed with serotype 1 cells; right well (py), antisera absorbed with serotype 3 cells. Note that all of the antibody was absorbed with the homologous serotype of cells, but only part with serotype 1 cells. *B:* Center well (P), same as in *A*; left well (l), antisera T-3 raised with infected tissue; top well (lx), T-3 antisera absorbed with serotype 1 cells; right well (ly), T-3 antisera absorbed with serotype 3 cells. Note that either cell type absorbed all of the antibody. *C:* Center well (L), saline extract of liver from turkey infected with serotype 3 *P. multocida*. Left and upper wells (p), AO-3 antisera induced with agar-cultured cells. Right and lower wells (l), T-3 antisera induced with infected tissues. Note that the extract from the infected liver produced a weak line of reaction with the T-3, but not with the AO-3 antisera.

T-3 antiserum did not actively cross-immunize turkeys, even though 100% cross-protection was induced with the tissue bacterin (Table IV).

Further evidence for antigenic differences between the agar-cultured bacterins (ACB) and the infected-tissue bacterins (TTB) was obtained by the gel-diffusion reactions of saline extracts of *P. multocida*-infected turkey livers. Weak lines of reaction were obtained with the liver extract and T-3 antiserum, but no lines of reaction were obtained with the liver extract and AO-3 antiserum (Fig. 1C).

Precipitins were isolated from the AO-3 and T-3 antiserum by ultracentrifugation and starch-block electrophoresis. Three protein peaks were found after electrophoresis (Fig. 2). Concentration of the eluates from the 1-cm fractions, and

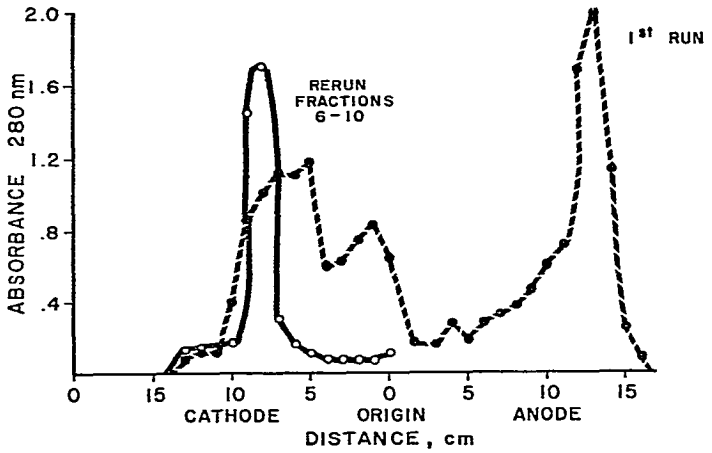

Fig. 2. Starch-block electrophoresis of turkey T-3 antiserum in pH 8.6 borate buffer, at 22° for 17 h, 15–10 mA at 300 V; each point represents 1-cm fractions after elution.

TABLE III

PASSIVE CROSS-IMMUNIZATION OF CHICKS WITH ANTISERA INDUCED WITH *Pasteurella multocida*-INFECTED TISSUE, BEFORE AND AFTER ABSORPTION WITH AGAR-CULTURED, *P. multocida* CELLS OF SEROTYPES 1 OR 3

Treatment of antiserum	*Number of survivors/number challenged*
Original T-3 antiserum[a]	8/14
T-3 antiserum, diluted and concentrated to original vol.	9/14
T-3 antiserum absorbed with serotype 1 cells	0/13
T-3 antiserum absorbed with serotype 3 cells	0/13
Normal serum for controls	0/14

[a]Serotype 3 antiserum (0.3 ml) was given s.c. in the neck, and 24 h later the chicks were inoculated with a challenge dose of 1000 *P. multocida* organisms of serotype 1.

TABLE IV

INDUCTION OF ACTIVE CROSS-IMMUNITY TO FOWL CHOLERA IN TURKEYS WITH *Pasteurella multocida* BACTERINS THAT WERE PREPARED WITH AGAR-CULTURED CELLS (ACB) AND *P.multocida*-INFECTED TURKEY TISSUE (TTB)

Bacterin	*Challenge strain*	*Number of survivors/ number challenged*[a]
Serotype 3 ACB[b]	Serotype 1	0/11
Serotype 3 TTB[c]	Serotype 1	11/11
Controls	Serotype 1	0/11

[a]All turkeys were given a challenge dose of 10,000 X-73F organisms of serotype 1, 1 month after the first inoculation. [b]P-1059B cells, serotype 3 (1.5 mg of dry metarial) in PBS (0.5 ml) were inoculated s.c. in 6-week-old turkeys; 2 weeks later, the same amount was given intramuscularly. [c]The formolized heart and liver suspension (1 ml) of a turkey that had died of exposure to *P. multocida* P-1059F, serotype 3, was inoculated s.c., then i.m., as with ACB.

evaluation of them with the serotype 3 endotoxin (2 mg/ml) in Ouchterlony plates gave lines of reaction with Fractions 6–10. These fractions were combined, concentrated, and fractionated by electrophoresis on a starch block as described earlier. All the precipitin activity was found in Fractions 8–9. Sedimentation-velocity determinations of the precipitins in PBS gave single peaks with the fractions from the AO-3 or the T-3 antisera, $S_{20,w}$ 6.6, 7.2, and 7.4 (Fig. 3). Electrophoresis on cellulose acetate in the Microzone apparatus (Beckman) gave single bands in the gamma region. The ultraviolet extinction coefficient at 280 nm of the T-3 precipitin fraction was $E^{1\%}_{1\,cm}$ 14.2 in PBS; the total protein was calculated by multiplying the nitrogen content by Kjeldahl with the factor 6.3.

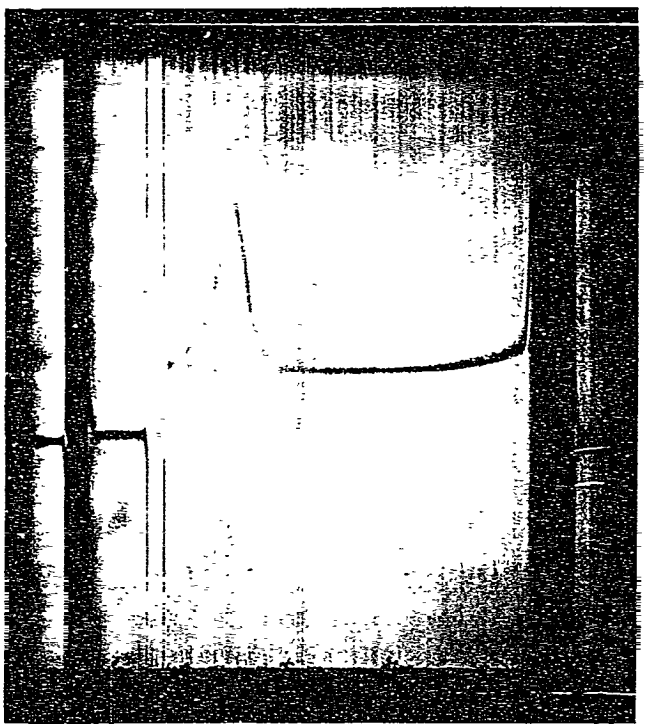

Fig. 3. Sedimentation velocity determination at 20° of turkey T-3 precipitin fraction, 7 mg/ml in PBS at pH 7.0, in Beckman Model E ultracentrifuge in double sector cell, after 20 min at 52,040 r.p.m.

The T-3 precipitin fraction was investigated by immunoelectrophoresis and the lines were developed with rabbit anti-turkey serum. Two lines were formed in the gamma globulin region (Fig. 4A). A sample of whole turkey serum was placed in the other well for comparison. In the second example, the T-3 precipitin fraction was compared with standard chicken IgG immunoglobulin (Fig. 4B). Although, in the experiment described in Fig. 4B, the lines were developed with rabbit anti-chicken

serum, almost identical patterns were obtained when the rabbit anti-turkey serum (described in Fig. 4A) was used. In the third example, the T-3 precipitins were compared with the chicken IgG immunoglobulin against an antiserum monospecific for chicken IgG immunoglobulin, as shown in Fig. 4C. The specificity of this

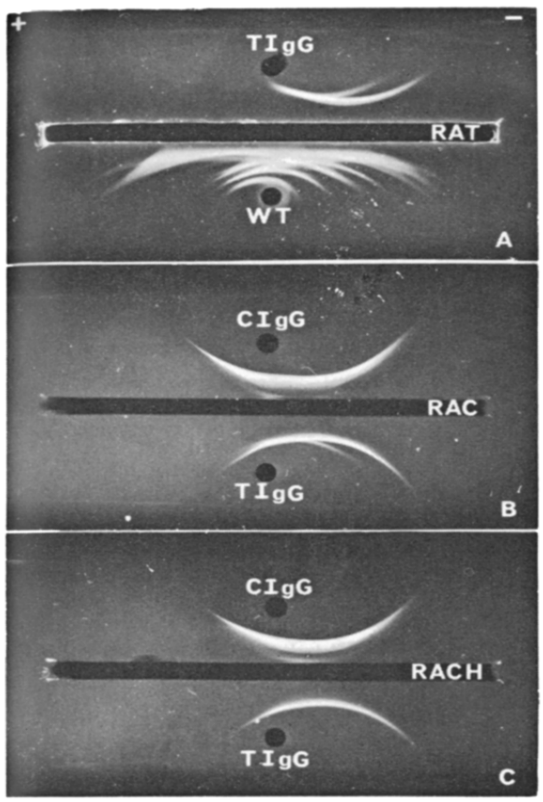

Fig. 4. Immunoelectrophoresis of turkey T-3 antiserum, 7S gel-precipitin fraction (TIgG). *A:* TIgG in upper well; in lower well, whole turkey serum (WT); trough filled with rabbit anti-turkey serum (RAT). *B:* TIgG in lower well; standard chicken CIgG in upper well; and trough filled with rabbit anti-chicken serum (RAC). No significant differences were obtained in the pattern when the trough was filled with rabbit anti-turkey serum. *C:* TIgG in lower well, and chicken CIgG in upper well. Trough filled with monospecific, rabbit anti-chicken, IgG antiserum (RACH). No differences in the pattern were obtained when whole turkey serum was added to the lower well and chicken serum to the upper well, when the trough contained the rabbit anti-chicken IgG antiserum.

antiserum (Fig. 4C) was evaluated by use of whole chicken and whole turkey serum as the antigens. Single lines were obtained with each serum. The T-3 precipitin fraction reacted very much like the chicken IgG immunoglobulin with this antiserum also.

The T-3 precipitin fraction was compared with that of the original T-3 serum in a passive cross-immunization experiment. The results given in Table V show that from

55 to 89% of the protective antibody present in the serum was isolated in this fraction. No conclusions can be drawn about the presence of protective antibodies in the other fractions.

TABLE V

PASSIVE CROSS-IMMUNIZATION OF CHICKS WITH ANTIBODY FRACTIONS OF T-3 ANTISERA AGAINST CHALLENGE INOCULATION WITH SEROTYPE 1 *Pasteurella multocida* ORGANISMS

Antiserum fraction	*Amount of serum (ml)*	*Number of survivors/ number challenged*
Whole turkey serum T-3, No. 7	0.3	9/9
Whole turkey serum T-3, No. 7, ultracentrifuged[a]	0.3	9/9
7S Fraction from T-3 No. 7 obtained by starch-block electrophoresis[b]	0.3	5/9
7S Fraction from T-3 No. 8 obtained by starch-block electrophoresis	0.3	8/9
Ditto	0.15	4/10
Control	0.0	0/11

[a]Serum centrifuged at 60,000 *g* for 17 h. A three-fold concentration of the gel precipitins was found in the lower 1/3 of the tube. The supernatant was discarded. The concentrated precipitin fraction was diluted back to the original volume of serum before testing. [b]The concentrated precipitin fraction was fractionated by electrophoresis on a starch block at pH 8.6 for 17 h at 22° at 300 V, and the fractions containing the precipitins were tested.

DISCUSSION

The results show that cross-immunity can be passively transferred to chickens or turkeys with the antiserum induced with infected-tissue bacterins, but not with antiserum induced with agar-cultured bacterins. One possible explanation for this behavior is that the antigenic components of the agar-cultured bacterins are not identical to those of the infected-tissue bacterins, and as a result, antibodies of different specificities are induced by the two bacterins. The free endotoxin preparations are able to induce type-specific immunity, and to give type-specific gel-diffusion reactions with the antisera from the immunized birds[2,3,5]. However, the quantitative and gel precipitin reactions of the antisera induced with the infected-tissue bacterins suggested that some antigen other than the type-specific free endotoxin was involved in the induction of the cross-immunity. The rather weak reaction of the antigen from the infected tissue with the infected-tissue antiserum supports, but does not prove, this point. The similarity of the antigens of the agar-cultured cells to those present in the infected-tissue bacterins was shown by the ability of the agar-cultured cells to absorb the cross-protective antibody. If the antigens had been identical, the agar-cultured cells should have been able to induce active cross-immunity, but they did not do so. The ability of the cells to absorb out the protective antibodies may be attributed

to serological cross-reactions, even though the antigenic determinants required for the induction of cross-immunity are incomplete.

When the *P. multocida* multiplies in living turkey tissue, we might expect the production of antigens that are different from those produced when cells are cultivated on laboratory media. For example, the influence of the media composition on the growth of *Bacillus anthracis* is well known; the protective antigen is elaborated from *in vitro*-cultured cells when the media contains serum and hydrogen carbonate ions[9]. Cultivation of *P. multocida* in defibrinated turkey blood at 42° has suggested that such bacterins can induce active cross-immunity in turkeys[1].

Starch-block electrophoresis of the T-3 antiserum gave good recovery of the cross-protective antibodies, and these were found to be in the 7S IgG immunoglobulin fraction. The gel-diffusion precipitin reaction used for monitoring may not have detected low concentrations of IgM or IgA antibodies, and as a result, we cannot say that the only antibodies in the T-3 antiserum are IgG immunoglobulins. Because the antiserum was collected 7 weeks after the first inoculation of antigen, it is not surprising that the major antibody is an IgG immunoglobulin. To the best of our knowledge, no other characterizations of turkey IgG antibodies or immunoglobulins have been published. For this reason, the fraction was compared with chicken IgG immunoglobulin by immunoelectrophoresis with rabbit anti-turkey serum, rabbit anti-chicken serum, and rabbit anti-chicken IgG heavy-chain serum. Strong cross-reactions were obtained with the turkey IgG fraction and the two rabbit anti-chicken sera. Workers have previously found that the IgG, but not the IgA or IgM immunoglobulins of chickens and turkeys cross-react[10]. Hence, the cross-protective antibody described herein is a turkey IgG immunoglobulin. The similarity of the turkey IgG to chicken IgG immunoglobulin is not surprising, the extensive cross-reactions of the serum components having been described[11].

The failure of the antiserum that passively immunizes chicks and turkeys to passively immunize mice agrees with results from previous reports[12–14]. Although one turkey antiserum[14] has been reported to passively immunize mice, the significance of this single experiment is not clear. The turkey antiserum was supplemented with fresh normal turkey serum to provide a source of turkey complement, but this did not improve the passive protection.

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