Production of Quellung Antisera in Chickens. (18028)

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The usefulness of the Neufeld capsular reaction for serological identification of certain microbial species is now generally recognized. The list of microorganisms for which preparation of type-specific antisera suitable for Quellung has been reported includes among others: *Diplococcus pneumoniae*(1), *Streptococcus salivarius*(2), *Streptococcus MG*(2), *Streptococcus agalactiae*(3), *Neisseria meningitidis* (4), *Hemophilus influenzae*(5), *Klebsiella pneumoniae*(3,6), *Bacterium antitratum*(7,8), *Sporotrichum schenckii*(9), *Cryptococcus neoformans*(10,11). In the case of the Gram-negative species, the preparation of such antisera in rabbits is often troublesome since prolonged courses of immunization may be necessary to ensure adequate antibody production and the toxicity of certain components of the bacterial cells often leads to premature death of the animals. In view of the ease with which antiserum for capsular swelling of meningococci can be obtained in chickens (12), it seemed worthwhile to attempt production of similar antisera versus *H. influenzae* type B and *K. pneumoniae* types A and B which are not uncommonly encountered in serious pathologic conditions and for which the diagnostic rabbit antisera are not always readily obtained from commercial sources.

Materials and methods. Preparation of Antigens for Injection. K. pneumoniae types A and B were cultured for 16 hours at 37°C on meat infusion agar and the growth emulsified in 0.9% saline containing 0.3% formalin. The same suspensions were used throughout the course of injections since the organisms retained their capsules unimpaired over several months on storage at 4-5°C. For injection the desired quantity of stock suspension, at a density of approximately 5 x 10^9 bacilli per ml, was diluted in saline to 2-5 ml.

H. influenzae type B (strain 62B) was subcultured weekly in fresh rabbit blood at 37°C for 16 hours; between passages the infected blood was stored at 4-5°C. To prepare antigen, the stock blood culture was streaked out on infusion agar containing 5% of Fildes' peptic digest of blood. After incubation overnight, the culture was checked microscopically for purity and re-streaked on a warm plate of Fildes' agar which was incubated for 6 hours at 37°C. The resulting growth was removed with a sterile cotton swab, emulsified in 0.9% saline, diluted to a turbidity corresponding to approximately 7-8 x 10^8 cells per ml (direct microscopic count) and the desired amount of this standardized suspension was further diluted to 2 ml with buffered saline pH 7.4. The suspensions were freshly prepared before each injection and checked for encapsulation by Quellung test.

Production of Antisera. Adult chickens of various breeds, weighing 5-7 lb, were used. They were injected in a wing vein and small bleedings were also taken from such veins. In the rabbits, weighing 5.6-6 lb, injections and small bleedings were made via an ear vein. In order to follow the rate of development of antibodies in the various animals

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TABLE I.
Production of Anti-*K. pneumoniae* Quellung Serum in Chickens.

Bird No.	Immunization schedule: Antigen employed	No. of injections	No. of bacteria per injection	Duration of course of injections	Interval between last injection and bleeding	Serum Quellung titer
	type		billions	days	days	
70	A	6	1.0-5.0	16	7	1:2
76	A	4	0.5-1.0	21	7	1:4
74	B	4	0.5-1.0	94	8	1:4
75	B	4	0.5-1.0	21	7	1:16

trial bleedings were made periodically, usually just prior to an injection of antigen. For large bleedings cardiac puncture was employed without anaesthesia; 30-50 ml was withdrawn without obvious harm to the animals of either species and on exsanguination up to 150 ml of blood was obtained from the birds. The blood was defibrinated with glass beads or allowed to clot spontaneously; serum was removed and stored with 0.01% merthiolate as preservative.

Capsular swelling tests. For *K. pneumoniae* the same stock suspensions of types A and B organisms used for the injection of animals were satisfactory for Quellung. For *H. influenzae* type B, 6-hour culture suspensions were freshly prepared and kept at 0°C during the period required to make the microscopic mounts. In each case the suspensions were adjusted so that the final mixtures would show 1-5 organisms per oil immersion field. The ability of the organisms to show Quellung was checked with commercial rabbit antiserum. Serial doubling dilutions of antiserum in saline were prepared; 2 loopfuls of a given dilution were mixed on a coverslip with a loopful of Loeffler's methylene blue solution and a loopful of bacterial suspension. Microscopic examinations of the moist preparations were made after the mixtures had stood at room temperature for 15 and for 30 minutes. For *H. influenzae* the various bleedings of the several animals were compared at the same time and the results checked by two competent observers.

Anti-K. pneumoniae serum. In Table I are summarized data on the production of antisera versus *K. pneumoniae* types A and B in roosters. In the first animal of the series, No. 70, 6 injections were made at intervals of 2-5 days and the dosage of organisms was higher than that employed for the later birds; serum collected 23 days after beginning the experiment gave excellent capsular swelling of the homologous antigen at dilution of 1:2. Bird 74 received 2 injections of *K. pneumoniae* type B suspension at an interval of 4 days; it was left for 81 days and then given a third dose. Five days later the animal was bled and the undiluted serum was found to exhibit Quellung. A fourth dose of antigen was administered 9 days after the third injection and the serum of the subsequent bleeding showed capsular swelling demonstrable at a dilution of 1:4. Roosters 75 and 76 each received 4 injections at weekly intervals and were bled 7 days after the last dose. The serum of bird 75 showed good capsular swelling versus type B organisms at dilutions as high as 1:16; rooster 76 serum was similarly active at 1:4 dilution versus type A capsular antigen.

Anti-H. influenzae serum. In this experiment an attempt was made to compare the

TABLE II.
Potency of Anti-*H. influenzae* Type B Serum Produced in Chickens and Rabbits.

Animal: Species	Sex	No.	Serum Quellung titer*
Chicken	♂	77	1:8
,,	,,	78	,,
,,	,,	81	,,
,,	♀	73	,,
,,	,,	80	,,
Rabbit	♂	315	undiluted
,,	,,	317	,,
,,	♀	314	,,

* Titer determined on bleeding taken 7 days after the fourth injection.

antibody response of chickens and rabbits when given the same quantities of *H. influenzae* type B, using suspensions of living organisms from young cultures. Each animal was given an initial dose of approximately 4 x 10^8 organisms followed by 3 or 4 injections of 8 x 10^8 cells each, at intervals of 7-8 days. The injections were well supported by the chickens, which showed no signs of illness. Among the 5 rabbits which were started in the experiment, 2 died following the first injection. The heart blood of one which was cultured at autopsy yielded a luxuriant pure growth of *H. influenzae*. It was not possible to autopsy the second rabbit promptly, so it was discarded.

One week after the second injection, 14 days after starting the immunization, 2 of the 3 chickens examined had already produced enough antibody to give definite capsular swelling with their undiluted serum. One week after the third injection, 20 or 22 days from the start, the serum of all 5 birds showed good Quellung at dilutions up to 1:8; no change in titer was observed after the fourth injection nor following a fifth injection made 8 to 10 days after the fourth, in the sera of 4 birds examined 8 to 12 days later.

By contrast, in the 3 rabbits which received the same course of injections, bleedings made after the fourth injection showed only a suggestion of capsular swelling even with undiluted serum and in the 2 animals from which a bleeding was obtained 8 days after the fifth injection, no increase in titer was noted.

Discussion. The notion that for Quellung tests rabbit antisera are inherently superior to those prepared in other species of animals has been shown to be erroneous. The determining factor is the quantity of anti-capsular antibody contained in the serum and equine antisera of comparable potency give capsular swelling as readily as those from rabbits(4,13). Our studies indicate that chickens likewise are eminently suitable animals for the production of capsular swelling antisera. While effective antisera have been obtained following various immunization schedules, we have found a procedure involving 3 or 4 injections separated by weekly intervals to be both rapid and convenient.

As regards the stability of chicken antibodies on prolonged storage, anti-meningococcal sera(12) preserved at 4-5°C have been tested 3½-4 years after their production and were found to have retained undiminished type-specific Quellung activity. Although we have not yet had opportunity to conduct tests with the anti-*H. influenzae* and anti-*K. pneumoniae* sera after similar periods of storage, our evidence to date indicates potency is maintained for at least a year.

Alexander(5), who introduced the capsular swelling test for the rapid diagnosis of meningitis due to *H. influenzae* type B, was able to produce rabbit antisera with an average Quellung titer of 1:10 by administering to her animals formalinized suspensions from 6-hour cultures in 3 series of 6 daily injections each with intervening rest periods of 8 days. The titer could be raised to 1:320 by prolonging the course of immunization to 24 weeks. In our experiment, antibodies adequate in concentration to give marked capsular swelling at serum dilutions up to 1:8 were regularly obtained in chickens after 3 or 4 injections of antigen, at a time when the corresponding rabbit sera were ineffective. The apparent superiority of chickens over rabbits for the production of antibodies reacting in other ways has been suggested by several investigators, among them Wolfe and Dilks(14,15) who emphasized the rapidity and regularity with which precipitating antibodies develop in chickens providing the latter are over 5 weeks old. Hilleman(16) has also pointed out the advantages of using chickens rather than rabbits for the production of neutralizing antibodies against the lymphogranuloma-psittacosis group of viral agents.

In addition to the ease and rapidity of production of antibodies in the chicken, the apparent innocuousness of *H. influenzae* for this species as compared with rabbits is note-

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worthy. In experiments directed toward the preparation of therapeutic anti-*H. influenzae* serum, Alexander and Heidelberger(17) found it necessary to extend the hyper-immunization over several months; they gave their rabbits multiple courses of injections each consisting of 4 daily doses of vaccine weekly for 4 weeks. A similar schedule was employed by Eaton *et al.*(18) for large-scale commercial production of antiserum. Both groups of workers noted(17,19) an appreciable mortality rate among the rabbits undergoing immunization. While some of the deaths were undoubtedly the result of the bleeding procedure and others were presumably due to intercurrent infections or other adverse conditions in the animal colonies, it is not unlikely that a portion of the deaths were due to the toxicity of the vaccines, since Dubos(20) has shown that these organisms may produce lethal soluble substance *in vitro*.

Our limited data on the preparation of anti-*K. pneumoniae* sera in chickens confirm those obtained for *N. meningitidis* and *H. influenzae*. It is highly likely that similar antisera can be readily prepared for other antigenic types of *Klebsiella pneumoniae* and *H. influenzae*, as well as various other encapsulated organisms. The successful results to date have been obtained with organisms the type-specific capsular antigens of which appear to be predominantly carbohydrate in nature. Whether Quellung would be demonstrable with non-carbohydrate capsular antigens is at the moment problematical.

Summary and conclusions. 1. Antisera satisfactory in the Quellung reaction were prepared for *K. pneumoniae* types A and B by injection of adult chickens with formalinized suspensions of the encapsulated bacilli. As few as 4 injections at weekly intervals were sufficient for this purpose.

2. Effective antiserum was similarly prepared for *H. influenzae* type B. Capsular swelling was readily demonstrable with sera collected from chickens after 3 or 4 weekly injections of living organisms from young cultures, while sera from rabbits undergoing the same immunization schedule were distinctly inferior at this stage.

3. Chickens are very useful for the production of Quellung antisera, with particular advantages where Gram-negative bacteria are concerned.

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Effects of Phosphorus^{32} on the Hamster. (18029)

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Phosphorus^{32} was used in therapy as early as 1936. Later, Lawrence, Scott and Tuttle (1) employed it in a study of chronic leukemia and recommended its clinical use. Erf(2) attested its use in treatment of polycythemia. At the present time it is the treatment of choice for many cases of polycythemia. Dosages of phosphorus^{32} sufficient to bring

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