

were performed on consecutive days.

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Properties of Mycobacteriophage DS6A

I. Immunogenicity in Rabbits* (33838)

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Mycobacteriophage DS6A was found by Redmond and Cater (1) and Redmond (2) used it to identify human strains of mycobacteria in phage typing of the genus. In edition, Mankiewicz and Béland (3) reported producing experimental sarcoid lesions by injecting phage DS6A and *Mycobacterium tuberculosis*, strain H₃₇Rv, into guinea pigs. They found neutralizing activity in 15 of the 16 sera tested (94%). The 16 sera had neutralizing activity against a heterologous unnamed phage in the same proportion (94%); and the neutralizing activity against a second heterologous unnamed phase was reported as 80%. Since Mankiewicz and Béland's neutralization experiments did not yield quantitative data (3), it seemed desirable to perform quantitative immunogenicity and neutralization studies with this phage. In addition, since the results of studying the immunogenicity of phages R1, D29, and Leo in rabbits were recently presented (4), a need existed for comparing the immunogenicity of phage DS6A with the three former phages. Accordingly, this report gives the results ob-

tained after injecting rabbits with mycobacteriophage DS6A, and the results of some cross neutralization experiments are presented also.

Materials and Methods. *TB broth.* For liquid cultures, the medium used contained the following ingredients: TB broth base (Difco), 11.6 g; glycerol, 10 ml; bovine serum albumin, fraction V (Armour and Company) 0.15 g; water 1000 ml. The pH was adjusted to 7.0 before autoclaving.

TB bottom agar. The TB broth was solidified with agar (Difco), 10 g/liter. After autoclaving, the medium was distributed in 30 ml amounts in sterile plastic petri plates.

TB soft agar. This consisted of 1 liter of TB broth with 7.0 g of agar. Before autoclaving, the medium was distributed in 3-ml amounts in test tubes. For use, the TB soft agar was melted at 100° and held at 44° for inoculation with phage and bacteria.

Mycobacteria. *Mycobacterium tuberculosis*, strain H₃₇Rv, was obtained from Dr. W. B. Redmond.² The BCG was obtained from a commercial vaccine preparation (Eli Lilly and Company). Stocks of each strain were maintained on Lowenstein-Jensen slants, with

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transfers of cells being made every 2-3 months. To obtain plating bacteria, cells were inoculated onto the surface of 100 ml of TB broth contained in flasks with approximately 100 glass beads (6 mm o.d.). The cultures were incubated at 37° and after about 2 or 3 weeks' growth, a pellicle formed. When the latter was about a week old the culture fluid was siphoned from the flask, leaving only cells and beads. The cells and beads were then shaken (two hundred 3-in. horizontal strokes/min for 15 to 20 min at room temperature) for homogenization of large clumps of cells. The cells thus separated were resuspended in broth to a concentration of approximately 60 mg/ml by the Hopkin's tube technique (5).

Mycobacteriophage DS6A. The phage was also obtained from Dr. W. B. Redmond.² Plate lysates of this phase were made on *M tuberculosis*, strain H₃₇Rv, on TB bottom agar. For this purpose about 5000 plaque-forming particles of phage DS6A were mixed with 1.0 ml of an H₃₇Rv suspension (described above) in melted soft agar and the contents then was poured onto the surface of a TB bottom agar plate and incubated at 37°. Plaques (confluent lysis) developed and were thought to be optimal for harvesting phage between 4 and 6 days. The phage in the soft agar layer was then collected by broth elution as previously described for mycobacteriophage D29 (6). The phage was then purified by differential centrifugation techniques as employed with phages R1, D29, and Leo (4).

Phage assays were performed using BCG as the plating bacteria. For this purpose, 0.05 ml of an appropriate dilution of mycobacteriophage DS6A was mixed with 1.0 ml of a BCG suspension (described above) in 3 ml of melted soft agar. The contents were then poured onto the surface of a TB bottom agar plate. After solidification of the agar the plates were incubated at 37° for 5-6 days. Plaques were counted with the aid of a Bactronic colony counter. Counts between duplicate plates generally agreed within approximately 10% of each other.

Production of antiphage antibodies in rab-

bbits. Mycobacteriophage DS6A was injected, at weekly intervals, into rabbits for 12 weeks. Two rabbits each were injected with 1.0 or 0.5 ml of broth suspended phage by the following three methods: subcutaneous; subcutaneous, with the phage suspension being mixed 1:1 with incomplete Freund's adjuvant (7); and intravenous. The concentrations of phage used were between 10⁸ and 10⁹ plaque-forming units/ml. Blood samples were taken from each rabbit by cardiac puncture at weekly intervals (except the first week following the initial injection). The blood was collected in sterile tubes and allowed to clot at room temperature, the clots were then ringed and the tubes were stored overnight in 4°. The following day the clotted blood was centrifuged at 1000g for 15-30 min and the sera were collected and stored at -20° in sealed tubes for subsequent testing.

Neutralization experiments. The stock phage was diluted in broth to a concentration 10 times greater than that to be used in the phage-antiphage reaction mixture (usually about 5 × 10⁷ plaque-forming units/ml). The rabbit serum was diluted in broth to the concentration to be used in the neutralization reaction mixture and prewarmed at 37°. One-tenth ml of the diluted phage preparation was then added to 0.9 ml of the prewarmed antibody solution and the mixture was incubated at 37°.

Then at intervals, the concentration of unneutralized phage in the reaction mixture was determined by removing a sample, diluting it 1:50 or 1:100 or top neutralization, diluting further if necessary, and plating a sample from the diluted mixture in duplicate or quadruplicate with BCG in TB soft agar. A control neutralization mixture consisted of 0.1 ml of the diluted stock phage mixed with 0.9 ml of broth incubated at 37°. Two 0.1-ml samples were removed from the control neutralization mixture, diluted, and plated in quadruplicate. This assay procedure on the control was repeated at the end of each experiment and the mean plaque count from the 16 plates was used to determine the input phage (P_0) concentration. The relative neutralization activity of each serum was calcu-

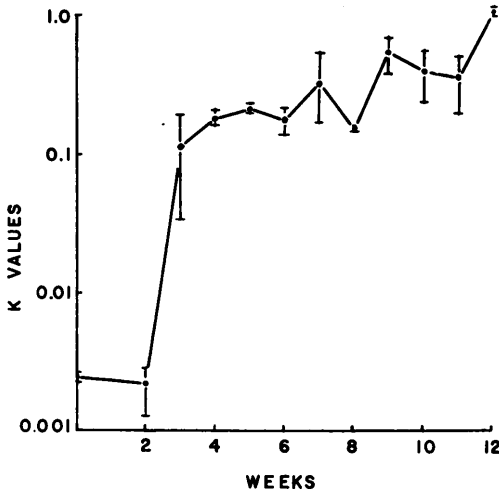


FIG. 1. Increase in K values of sera from two rabbits injected subcutaneously with purified mycobacteriophage DS6A in broth at weekly intervals: the curve is drawn through the mean K values.

lated in terms of their K values. The latter was determined by use of the formula

$$K \times 2.3 D/t \times \log P_0/P,$$

where D represents the final dilution of serum in the phage-serum mixture, t is time and P is the surviving phage concentration at t . The K value of a given serum is an estimate of its relative antibody content.

Results. Antibody production to mycobacteriophage DS6A. Figure 1 shows the rate and extent of the immunogenic response of rabbits injected at weekly intervals with mycobacteriophage DS6A subcutaneously in broth. As shown, following the first three injections there occurred an approximately 100-fold rise in K values between the second and third weeks of treatment. During the subsequent 9 weeks of treatment there was only one further 10-fold increase in K value. The highest K value obtained was approximately 1.0 and this was seen only in serum obtained from the twelfth-week bleeding.

The rate and extent of the immunogenic response of rabbits injected at weekly intervals with phage DS6A subcutaneously with Freund's incomplete adjuvant is shown in Fig. 2. As shown, between the second and third weeks of treatment the K values in-

creased (approximately logarithmically) for 5 subsequent weeks to a value about 1000 times greater than that observed initially. There was no significant increase or decrease in K value during the last 5 weeks of treatment. The total immunogenic response was seen to be presented by K values in the vicinity of 10.

The extent of neutralizing capacity of sera from rabbits injected intravenously with mycobacteriophage DS6A at weekly intervals and the rate of this immunogenic response is shown in Fig. 3. As shown, the initial K values were between 0.01 and 0.001 and no significant increase occurred during the first 6 weeks. For the next 4 weeks the K values increased by a factor of 10 to 30. Subsequently, there was a 14-week interruption in the injection and bleeding schedules. Upon resumption, as shown, there occurred only a slight anamnestic response (K values increased slowly only by a factor of 10). Shown also is the level of the final extent of neutralizing activity at a K value of approx-

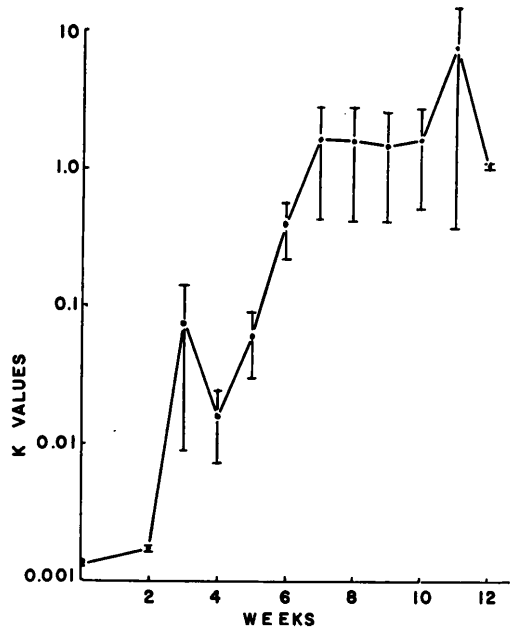


FIG. 2. Increase in K values of sera from two rabbits injected subcutaneously with purified mycobacteriophage DS6A in incomplete Freund's adjuvant at weekly intervals: the curve is drawn through the mean K values.

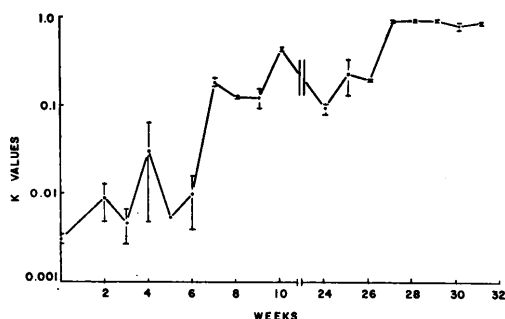


FIG. 3. Increase in K values of sera from two rabbits injected intravenously with purified mycobacteriophage DS6A in broth in weekly intervals: A 3-month interruption occurred between weeks 10 and 24; the curve is drawn through the mean K values.

imately 1.0. No significant change in K values was seen during the last 4 weeks of treatment.

In a preliminary study to determine if some of the above neutralization observed was due to the IgM type of antibody, the serum obtained from the terminal bleeding of one animal (last point, Fig. 3) was treated with 2-mercaptoethanol for 24 hrs (8) and then tested for its neutralizing activity. The results indicated that this treatment inactivated approximately 20% of the serum's neutralizing activity against phage DS6A. Control serum treated with phosphate buffer lost none of its neutralizing activity.

Serological relatedness of mycobacteriophage DS6A to mycobacteriophages R1, Leo and D29. Serum obtained from the penultimate bleeding of one of the rabbits (Fig. 2, eleventh week, top point) was used in neutralization experiments with phage DS6A and with other mycobacteriophages. Also other mycobacteriophage sera were tested against phage DS6A. Table I gives the results and it shows that anti-DS6A antiserum had no neutralizing activity against mycobacteriophages D29, Leo, and R1. In the same fashion, anti-R1, anti-D29, and anti-Leo sera had little or no neutralizing capacity for mycobacteriophages DS6A.

Discussion. Mycobacteriophage DS6A is immunogenic in rabbits but poorly so. Its immunogenicity by the intravenous or subcu-

taneous (without incomplete Freund's adjuvant) routes of injection appears to be approximately equal. However, its immunogenicity was enhanced slightly when given subcutaneously with Freund's incomplete adjuvant. This apparent enhancement of immunogenicity was not seen in a previous study (4) with mycobacteriophages D29, Leo, and R1 in rabbits. By any route used, the extent of antibody produced (highest K values) to mycobacteriophage DS6A during 3 months (or more) of weekly injections was not greater than 20. This value is about 5 times less than that obtained with mycobacteriophages R1, Leo, and D29 (4); or about 150 (9) to 200 times (10) less than that obtained with phage ϕ C174 in rabbits after only two to four injections. The poor immunogenicity of phage DS6A might be related to the fact that it apparently contains essential lipids, since phage DS6A is inactivated by chloroform treatment (11).

An interesting observation was made on the rabbits' response during their course of treatment with mycobacteriophage DS6A when given intravenously. Rabbits reinjected with the phage at weekly intervals following a 3-months' pause in a previous series of injections showed no significant anamnestic response (Fig. 3). This finding coupled with the preliminary finding that the neutralizing activity of this serum was partially inactivated by 2-mercaptoethanol suggests that the terminal serum contained both IgM and IgG classes of antibody. This latter observation is at variance with findings of Uhr *et al.* (12) with bacteriophage ϕ X174 and of Sve-

TABLE I. The K Values of Sera in Homologous and Heterologous Mycobacteriophage-Antiphage Systems.*

Antiserum to phage	Phage			
	D29	Leo	R1	DS6A
D29	145.2	—	—	0.0
Leo	—	32.3	—	0.0
R1	—	—	37.4	3.5
DS6A	0.0	0.0	0.0	14.8

* DS6A serum used diluted 1:5 in broth; all other sera used diluted 1:1000 in broth.

hag and Mandel (13) with poliovirus. They reported the IgM (19S) type of antibody to be present only in the early immune response sera and not in late sera.

Since antiserum to phage DS6A did not neutralize significantly bacteriophages D29, R1, or Leo, this serum appears to be specific for phage DS6A (Table I). The conclusion may also be made that mycobacteriophages DS6A, D29, Leo, and R1 are serologically unrelated in neutralization tests.

Summary. The antigenicity of mycobacteriophage DS6A was determined in rabbits. The concentration of antibody produced was low (*K* values 10 or less). The amount of antibody produced appears to be dependent on the method of immunization. Bacteriophage DS6A appears to be serologically unrelated to mycobacteriophages D29, R1, and Leo by neutralization tests.

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Collagen Biosynthesis in Normal and Cirrhotic Rat Liver Slices* (33839)

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The fibrosis that accompanies hepatic cirrhosis, either that produced in animals experimentally (for example, by CCl₄, ethionine, or choline-free diet) or that occurring in man (viral, alcoholic-nutritional or biliary) is one of the main features of the disease.

Two mechanisms have been proposed for the development of fibrosis: (a) condensation or collapse of preexisting hepatic stroma (2), and (b) induction of fibroblastic proliferation and *de novo* collagen deposition (5). The latter mechanism is supported by evidence showing a net increase of total collagen

content or an increase of hydroxyproline content per unit weight of liver (3, 7). The present experiments were designed to explore the capacities of normal and cirrhotic rat liver slices to synthesize protein containing radioactive hydroxyproline in the course of incubation with labeled proline.

Materials and Methods. Male Wistar albino rats weighing from 60 to 70 g were fed *ad libitum* with Purina chow. Cirrhosis was produced by intraperitoneal injection, 3 times/week, of 0.15 ml of a 1:7 solution of CCl₄ in mineral oil; each rat received a total of 20 injections.

Two days after receiving the last injection, a rat was deprived of food but not of water during 16 hr. It was stunned by a sharp blow to the head, decapitated, and the liver was removed to ice-cold Ringer-NaHCO₃ buffer.

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