infections, their role in apparently non-specific resistance should be considered along with properdin and other factors.

Summary. Two modifications of a staphylococcal hemagglutination test, utilizing protease for treatment of erythrocytes or Coombs' antiserum, are described and are more sensitive than the basic hemagglutination method for titration of staphylococcal antibodies in human gamma globulin and serum. These antibodies probably are directed against an antigen of the Rantz type, as the antigen is heat stable at acid and labile at alkaline pH and since both *Staphylococcus aureus* and *B. subtilis,* in contrast to *E. coli* and *Staphylococcus citreus,* remove antibodies from human gamma globulin. The significance of the data is discussed.

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New Observations on Pneumococcal Transformations *in vivo.** (24991)

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The first genetic transformation of pneumococcal types was reported by Griffith(1), who inoculated mice subcutaneously with a mixture of live unencapsulated pneumococci and a vaccine of heat-killed encapsulated pneumococci. The mice developed an infection due to encapsulated pneumococci of the same type as the strain from which the vaccine had been prepared. Since that time most of our information concerning genetic transformation of bacteria has come from studies *in vitro,* although there has been some extension of Griffith's technic *in vivo,* as for example by Austrian(2). We were interested in using purified deoxyribonucleic acid (DNA) preparations for transforming experiments *in vivo.* Previously, only vaccines of intact cells had been used. This report describes results of using partially purified DNA preparations *in vivo,* as well as a modification of Griffith's original technic.

Methods and materials. 1. Strains of *Diplococcus pneumoniae.* SV1, Type I strain virulent.‡ A-66, Type III strain virulent. A-66 1,000, Type III, a mutant obtained from strain A-66 in one step, resistant to 1000 γ/

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‡ Virulence of pneumococci seems to depend directly on amount of polysaccharide capsule(3).

ml of streptomycin. R36NC, an avirulent strain of pneumococcus originally obtained from fully encapsulated Type II D39S strain. It is sensitive to less than 1 γ/ml of streptomycin.

Virulent strains were maintained by weekly passages in mice according to following protocol. One ml of stock culture was transferred to 9.5 ml of fresh blood trypticase soy broth (blood-broth) and incubated 5 to 6 hours at 37°. The culture was diluted to 10^{-4} in trypticase soy broth and 0.5 ml of the diluted solution was injected intraperitoneally into each of 2 mice. The mice were sacrificed 24 hours later and heart blood passed into blood-broth, which was incubated and stored. Blood agar plates were routinely streaked with heart blood of each animal to check for contamination and level of resistance to streptomycin. Swiss female mice, 16-20 g, obtained from Carworth Farms (CF-1) were used.

Vaccine. The procedure of MacLeod and Kraus(4) was used for growing pneumococcus in quantity. We standardized our preparations by growing each culture until it had taken up 20 ml of 3N sodium hydroxide/liter and then concentrating the cells to 20 ml for each liter of original culture. Vaccines were prepared according to procedure of above authors(4) which essentially consisted of heating fresh concentrate of cells at 65° for one-half hour. All preparations were routinely checked for sterility both *in vitro* and *in vivo*. No vaccines were ever contaminated with live organisms.

Results. 1. *Standard transformation procedure* in vivo. The technic employed was patterned after Griffith's original procedure (1) further modified by MacLeod and Kraus (4). For consistent results it was necessary to use a fresh culture of strain R36NC. One ml of fresh overnight culture of strain R36NC in blood-broth was passed into 10 ml of blood-broth which was then incubated 5 to 6 hours at 37°. The culture was used immediately. One part of culture was mixed with 4 parts of vaccine and 0.5 ml of the mixture was immediately injected subcutaneously into the lower abdominal region of mice. This procedure resulted consistently in nearly 100% mortality of animals that received vaccines prepared from all 3 virulent strains (Table I).

TABLE I. Standard Transforming Procedure (Transfer of Virulence Marker).

Treatment	No. of mice	Total dead 7 days post treatment
SV1 vaccine mixed with R36NC	99	97
" " alone	70	0
A-66 vaccine mixed with R36NC	30	25
" " alone	20	0
A-66-1,000 vaccine mixed with R36NC	20	19
A-66-1,000 vaccine alone	10	0
R36NC culture alone	80	2

Separate groups of mice were inj. (s.c.) with 0.5 ml of a mixture of vaccine and fresh R36NC culture (4:1), 0.4 ml of vaccine and 0.2 ml of fresh R36NC culture.

Lysates of virulent strains. Lysates of all 3 virulent strains were prepared. Physically the results were similar but biologically Type III strain proved most successful. The following procedure used for all 3 strains is described in terms of strain A-66. One hundred ml of concentrated live cells obtained as described above was placed in 125 ml Erlenmeyer flask immersed to its lip in water bath at 65°. The mass was stirred gently with a paddle stirrer for 15 minutes, then 5 ml of fresh 10% sodium deoxycholate solution (aqueous) was added and stirring continued a further 10 minutes while temperature was maintained at 65°. Microscopic examination of the lysate at this point showed extremely few intact cells. The lysate was chilled and 3 volumes of cold 95% ethanol were added with stirring. The precipitate was collected in centrifuge at 2,000 rpm for 20 minutes, blotted with filter paper and resuspended in 100 ml of buffer (0.14 M NaCl, 0.005 M $MgCl_2$, 0.003 M $CaCl_2$, pH 7.1). The fibers were dispersed by forcing the suspension through a No. 24 needle. The suspension was stirred one hour at 0°, then centrifuged for 45 minutes at 27,000 g. The first supernatant was very viscous and opalescent. The procedure was repeated twice more and the residue homogenized in 100 ml of buffer by repeated extrusion through No. 24 needle. This suspension is called A-66 residue. All fractions of the above protocol were analyzed for DNA(5) and carbohydrate(6). The first

TABLE II. Comparison of a Purified Preparation with Vaccine in Standard Transforming Procedure.

Treatment	No. of animals	Total dead 7 days post treatment
A-66 vaccine diluted 20 × with saline mixed with R36NC	10	7
A-66 vaccine alone	5	0
A-66 residue mixed with R36NC	40	39
A-66 residue diluted 20 × mixed with R36NC	10	7
A-66 residue diluted 100 × mixed with R36NC	10	5
A-66 residue alone	40	0
R36NC culture alone	20	0

Separate groups of mice were inj. (s.c.) with 0.5 ml of a mixture of DNA source (diluted or undiluted with physiological saline) and fresh R36NC culture (4:1), 0.4 ml undiluted DNA source alone, and 0.2 ml of fresh R36NC culture alone.

homogenate, the ethanol precipitate in the buffer solution contained 2500 γ/ml of DNA and 1700 γ/ml of carbohydrate. The resuspended A-66 residue contained 2200 γ/ml of DNA and 660 γ/ml of carbohydrate. Practically all the sugar was removed in the first wash. The A-66 residue had as much transforming activity as a comparable volume of heat-killed vaccine prepared from the A-66 strain (Table II). It could be stored at 4° for at least 2 months without losing transforming activity. The results were very reproducible. Six separate runs gave same results each time. The infecting organisms resulting from transforming experiments in which the A-66 residue was used were isolated as follows. Twenty mice received a single subcutaneous injection of a mixture of 4 parts A-66 residue and one part of a culture of strain R36NC. Forty-two hours later all mice were very sick. Ten were sacrificed. The remaining mice died within 2 days. Heart blood of each sacrificed animal was passed into blood-broth tubes. After overnight incubation 8 of 10 tubes contained a bacterial growth, which in each case was due to a Type III pneumococcus. Virulence of the 8 cultures was established by injecting intraperitoneally 0.5 ml of a 10^{-6} dilution of fresh cultures into mice (10 mice for each culture). Within 48 hours, 70 of the 80 mice died. Four of the remaining mice (all sick) were sacrificed and the infecting organism was isolated. It was in each case a Type III pneumococcus.

The residue prepared from the SV1 strain according to above procedure gave similar chemical analyses indicating that most of the original DNA content of the cells was still in the residue. But at best, this residue had weak transient activity in the standard transforming system. Results were inconsistent over several runs; in general this residue was inactive.

Inactivation of A-66 residue with detergent or chloroform. Twenty ml of the A-66 residue was shaken vigorously with 20 ml of chloroform and 0.6 ml of *iso*amyl alcohol for 5 minutes. The aqueous layer was immediately assayed for transforming activity in the *in vivo* system and found to be inactive. Twenty ml of A-66 residue was stirred 3 hours at 0° in presence of 2 ml of 5% dodecyl sulfate solution. The solution was centrifuged at 27,000 g. for 20 minutes, after which 3 volumes of ethanol were added to the clear supernatant. The fibrous precipitate was immediately homogenized in 20 ml of normal saline and assayed in the standard transforming system. It was completely inactive.

Transformation of streptomycin sensitive pneumococci into resistant pneumococci. Twenty mice were injected subcutaneously with 0.5 ml of a 1:4 mixture of a fresh culture of R36NC strain and residue from strain A-66-1,000 obtained in same manner as A-66 residue. Seven hours later, 10 mice were injected subcutaneously with 400 mg/kg of streptomycin sulfate (data in Table III show that a 7 hour post-infection administration will protect about 80% of mice infected with the SV1 strain). Nine of the 10 mice not re-

TABLE III. Protection by Streptomycin, of Mice Infected with SV1 Strain of Pneumococcus.

Hr post infection single dose (s.c.) of 400 mg/kg of streptomycin sulfate	Total mortality 6 days post infection
0	0/10
7	2/10
16	9/10
24	10/10
40	10/10
No streptomycin	10/10

Mice were infected with 0.5 ml of a 10^{-6} dilution of a fresh 6 hr culture of SV1 pneumococcus.

ceiving streptomycin died within 3 days post infection. The 10 mice which received streptomycin were sacrificed 42 hours post infection. Heart blood from each animal was streaked on blood agar plates. After 24 hours incubation, the 10 plates were covered with smooth glistening colonies. The plates were replicated(7) to blood agar plates containing 0, 5 and 1,000 γ/ml of streptomycin sulfate. All 10 isolates showed equal number of colonies on all 3 replicates. A similar experiment starting with an A-66 vaccine resulted in no colonies that would grow on blood agar plates containing 20 γ/ml of streptomycin sulfate.

Variation on Standard Transformation Technic in vivo. Vaccines from strains SV1, A-66, and A-66-1,000 or the A-66 residue were injected subcutaneously *per se* and the avirulent strain R36NC was injected intraperitoneally *per se*. This modification was about 70% as effective as the classical procedure judging from mortality of animals (Table IV). The R36NC culture did not have to be injected simultaneously with the vaccine injection; indeed, successful transformation still occurred when injection of the R36NC culture was delayed 24 hours after vaccine injection (Table V).

Discussion. Many workers have used Griffith's technic for transformation of avirulent pneumococci to virulent pneumococci *in vivo*. We used his technic essentially as a control for biological activity of the vaccine and lysate preparations. By paying close attention to the condition of the avirulent culture (R36NC), nearly 100% mortality of treated

TABLE IV. Modified Transforming Procedure *In Vivo*.

Treatment	No. of mice	Total dead 7 days post treatment
SV1 vaccine + R36NC	54	35
SV1 vaccine	10	0
A-66 " + R36NC	10	6
" residue + "	10	6
" vaccine	10	0
" residue	10	0
R36NC	10	0

Mice received 0.5 ml of vaccine or residue subcut. and 0.2 ml of fresh R36NC culture intraper. Control groups received only a single inj. of either the transforming factors or R36NC culture in the same amount.

TABLE V. Transfer of Virulence Marker *In Vivo* by Modified Transforming Procedure.

Hr between administration of SV1 vaccine (s.c.) and R36NC culture (i.p.)	Total dead 5 days post treatment
0	8/10
4	8/10
8	10/10
24	10/10
Vaccine alone	0/5
R36NC alone	0/5

Separate groups of mice were inj. (s.c.) with 0.5 ml of 1:1 dilution of SV1 vaccine prior to inj. (i.p.) of all groups at the same time with 0.2 ml of a fresh 6 hr culture of R36NC.

groups was consistently obtained. The results also showed that amount of vaccine necessary for successful transformation could be reduced at least 50-fold from that used by Griffith.

Injection of an animal with a mixture of transforming material (vaccine) and an avirulent culture subcutaneously, results in a pocket where intimate contact between components of mixture is maintained for considerable time before complete absorption by host. Transformation could occur during this time which could hardly mean true involvement of host in the process. This objection was met by injecting the transforming material and live avirulent culture separately at different sites in the animal. The positive results of this technic mean that interaction of the 2 components of the transforming system was mediated by circulatory action within the living host. Transformation of pneumococcal capsule types *via* this technic, although not as efficient as *via* the standard procedure was achieved with consistent results with several batches of vaccines and DNA lysate preparations. The technic of separate injections affords a method for studying survival of biologically active DNA in the living animal. In one experiment vaccine prepared from the SV1 (Type I) strain was injected 24 hours prior to injection of the live avirulent cells. The treated animals died indicating that DNA with transforming activity in this system survived *in vivo*, at least 24 hours.

One of the main objectives of this program was to make purified DNA transforming

preparations that would be active *in vivo*. Certainly, for *in vitro* transforming systems, highly purified DNA samples can be successfully used. Austrian tried a purified, relatively protein-free DNA preparation, that would have had transforming activity *in vitro*; but it was inactive *in vivo*. First steps toward an active *in vivo* preparation were taken by heating fresh live cells in presence of deoxycholate, similar to procedure used by Alloway for DNA preparation used *in vitro*(8). This procedure essentially accomplished 3 things: lysis, sterility, and freedom from nucleases. The lysate was diluted with ethanol and the resultant precipitate washed twice with dilute buffer. Practically all the original DNA remained in the final residue. Physical and chemical response of the 2 strains, SV1 and A-66, to this procedure was similar. Only the residue from the Type III strain (A-66) was active in the *in vivo* transforming system. In fact this preparation was as active as a comparable amount of vaccine prepared from same strain. It could be stored in aqueous suspension at 4° for several weeks without impairment of activity. There was no apparent reason for lack of parallel activity in the Type I preparation.

The partially purified transforming principle from the Type III strain was inactivated by mild protein denaturation caused by either the chloroform procedure or detergent procedure. It is well known that such procedures are not deleterious to *in vitro* transforming activity of DNA preparations(9,10). The loss of transforming activity *in vivo* caused by these procedures might infer that the protein conjugate is required for stability of DNA in an *in vivo* system; the suggestion may be that the physical structure of DNA must be more intact for biological activity in an *in vivo* system than in an *in vitro* system.

Streptomycin resistance can be conferred on a streptomycin sensitive pneumococcus *via* a transforming preparation *in vivo*. Actually, the experimental results described here depended on double transformation of both virulence and streptomycin resistance markers to the recipient R36NC culture. The transformed bacteria were resistant to 1000 γ/ml of streptomycin sulfate, the resistance of donor culture, which was a "one-step" mutant of strain A-66. Langvad-Nielsen performed a similar experiment but omitted the streptomycin injection with the result that only the virulence marker was transferred(11). Austrian has reported a double transformation *in vivo* of type specific capsular polysaccharide and type specific M protein(2).

Summary. 1. Succesful pneumococcal transformation *in vivo* has been achieved by injecting the transforming preparation subcutaneously and the live avirulent culture intraperitoneally. 2. The transforming activity of a vaccine preparation lasted *in vivo* at least 24 hours. 3. Transforming preparations active *in vivo* have been prepared from a lysate of a culture of strain A-66 (Type III). This preparation was inactivated by mild protein denaturation. 4. The genetic marker of streptomycin resistance was transferred between pneumococcal strains *in vivo*.

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