

Immuno-Stimulation by a Ribosomal Vaccine Associated with a Bacterial Cell Wall Adjuvant in Humans

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We have studied a new vaccine of ribosomal nature associated with glycoprotein cell walls from *Klebsiella pneumoniae* which served as an immunoadjuvant. This vaccine was administered by the aerosol route to working men free of any important disease, especially of respiratory disease. A total of 104 men working for the Commissariat à l'Energie Atomique, all volunteers, were randomly placed into two groups. During the first period, 51 patients (group I) were vaccinated three times a week during 5 weeks, and the second group was used as control. During the second period, which started on day 225, the control group received the vaccine, and the first group was revaccinated. Results of this experience show a significant difference in the immunity of the two groups. The specific antibodies increased with vaccination as illustrated by chi-square test (Yates correction), which corresponds to an independent probability equal to 0 ($P = 0.5 \times 10^{-4}$).

Since the work of Youmans and Youmans (40, 41) on ribosomal fractions of *Mycobacterium tuberculosis*, numerous authors have studied the protection conferred in animals by subcellular fractions of various bacterial strains, such as *Salmonella typhimurium* (33) or *Vibrio cholerae* against a homologous or heterologous infection (1). Other ribosomal fractions or ribonucleic acid (RNA) were obtained from extra- or intracellular growth bacteria (*Streptococcus pneumoniae* [30; H. C. W. Thompson and I. S. Snyder, Program Abstr. Annu. Meet. Am. Soc. Microbiol. 74th, Chicago, Ill., abstr. no. 117M, 1974], *S. pyogenes* [25], *Neisseria meningitidis* [29, 35]). Dussourd d'Hinterland et al. (L. Dussourd d'Hinterland, F. B. Michel, and R. Fontanges, *Arzneim. Forsch.*, in press; L. Dussourd d'Hinterland, G. Normier, A. M. Pinel, and H. Serre, French patent Pierre Fabre S.A. 2.305.990, March, 1976; L. Dussourd d'Hinterland, G. Normier, A. M. Pinel, and H. Serre, B.O.P.I. Abstr. no. 49, p. 12, 179, 1976; L. Dussourd d'Hinterland, H. Serre, and G. Normier, French patent Pierre Fabre S.A. 2.253.499, August, 1975; L. Dussourd d'Hinterland, H. Serre, and G. Normier, B.O.P.I., Abstr. no. 32, p. 9, 353, 1975) studied extracts derived from pathogenic bacteria of the respiratory tract of animals (*S. pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and *S. pyogenes*).

All of these results combine to give to ribosomal fractions or RNA a vaccinal activity at least equivalent to the best attenuated vaccine,

either in immediate or long-term protection. Moreover, these vaccines are atoxic.

The effectiveness of these vaccines is further increased by the adjuvants which can be associated with them. The work of Youmans and Youmans (39) on *M. tuberculosis* and of Thompson and Snyder (30) on *S. pneumoniae* have shown their necessity in animals. On the other hand, the ribosomes of *S. typhimurium* (13, 32), of *N. meningitidis* (29), and of *Staphylococcus aureus* (38) appear to give a high degree of immunity without adjuvant.

Freund adjuvant, either complete or incomplete, is widely used in animals but is medically unacceptable in man. The glycopeptide adjuvants isolated by Adam et al. (2), Hiu (9), and Migliore-Samour and Jolles (18), now well known, have a high activity in vivo (2) because they potentiate both the humoral and cellular immunity without any toxicity (23).

Numerous substances reputed to be immunostimulants have been suggested as capable of preventing human bacterial respiratory infection, but we have little information about them in respect to (i) the choice of medication with an optimal capacity of immune stimulation, (ii) the method of their administration to those subjects needing treatment, or (iii) the criteria of their effectiveness.

To overcome, at least partly, these difficulties, a study based on the administration of a new type of vaccine (D 53) was carried out. It concerns a ribosomal vaccine supplemented with *K.*

pneumoniae cell wall glycoproteins as adjuvant.

Patients suffering from chronic respiratory infections were purposely not studied because, quite frequently, those infections cause irreversible anatomical lesions which make any objective investigation unreliable. We deliberately did not take into account clinical criteria because they are difficult to measure, and besides, they are not the best parameters of vaccine effectiveness. We studied the emergence of specific serum antibodies together with their evolution after vaccination and after booster doses of vaccine.

MATERIALS AND METHODS

Ribosome preparation. Ribosomes were extracted from a bacterial biomass obtained by cultivation in a fermentor.

The bacterial cells were mechanically disrupted under pressure in a Manton-Gaulin homogenizer at low temperature. The suspension was then submitted to a first centrifugation at $7,500 \times g$ to remove unbroken cells. The $7,500 \times g$ supernatant was then centrifuged for 30 min at $30,000 \times g$ and then for 20 min at $50,000 \times g$ at 4°C .

The ribosomes were then sedimented from the $50,000 \times g$ supernatant by a 2.5-h centrifugation at $140,000 \times g$ at 4°C . The ribosome pellet was suspended and washed for 1 h by sodium dodecyl sulfate at 25°C , and the ribosomes were again pelleted at 15°C (the sodium dodecyl sulfate being soluble at this temperature).

The ribosomes were next suspended at 0°C and purified by a last centrifugation for 20 min at $50,000 \times g$.

The supernatant containing ribosomes was sterilized by filtration and freeze-dried.

The ribosomal fraction was controlled by an RNA, a total protein, and a carbohydrate dosage (L. Dussourd d'Hinterland et al., French patent Pierre Fabre S.A. 2.305.990, March, 1976; Dussourd d'Hinterland et al., B.O.P.I., Abstr. no. 32, p. 12, 179). This fraction contained at least 70% of RNA. The peptidoglycan fraction was tested by taking into account several criteria: an RNA research, a dosage of proteins, amino acids, carbohydrates, hexosamines, and uronic and sialic acids. It was free from RNA or proteins (Dussourd d'Hinterland et al., French patent Pierre Fabre S.A. 2.305.990, March, 1976; Dussourd d'Hinterland et

al., B.O.P.I., Abstr. no. 32, p. 12, 179).

Vaccine. The formula for one vaccinal dose of D 53 was, in micrograms: ribosomes of *K. pneumoniae*, 3.5; ribosomes of *S. pneumoniae* type II, 3.0; ribosomes of *S. pyogenes* A₁₂, 3.0; ribosomes of *H. influenzae*, 0.5; and membranal glycoproteins of *K. pneumoniae*, 15.0.

Subjects vaccinated. One hundred and five healthy employees of the Commissariat à l'Energie Atomique of Pierrelatte, France, were involved. All were volunteers and male adults; they were divided into two groups (54 controls and 51 treated) as harmoniously as possible, obviously without knowledge of their immune status.

Vaccination procedure. Two vaccination series were done. The first lasted 5 weeks. D 53 was administered to the subjects of the treated group in the form of aerosols three times a week. Thus, 15 sessions were conducted using four doses a time. A total of 60 doses was given. The aerosolization sessions were conducted by a technique recommended by Fontanges and Fournier (7) with a generator (generosol) which features an aspiro-compressor unit and 150-W motor. This generator worked at a flow rate of 20 liters/min and at an air pressure under 1.5 kg for a maximum of 10 min. A booster sequence was carried out for 1 week, 15 days after the end of the first sequence.

The second series was carried out 6 months later. Most of the volunteers of the first period (99 out of 104) agreed to continue. Controls or treated subjects were submitted to a new sequence of D 53 vaccination administered in the form of a spray with double puff four times per day for 15 days. The spray was delivered by a special device providing $25 \mu\text{g}$ of immunizing particles. The size of each one was between 1 and $3 \mu\text{m}$. A total of 120 doses was administered. An immunological check was done before the booster and 30 days later (Fig. 1).

Clinical supervision. There was daily medical supervision during the first series. During the second series, each subject noted daily clinical manifestations or those related to a possible intolerance to the product.

Immunological investigations. This was investigated on days 0, 90, 225, and 255 and included (i) the dosage of immunoglobulins (immunoglobulin A [IgA], IgG, and IgM) by radial diffusion (Mancini technique) on Hyland immunoplates by comparison with a reference range, and (ii) the study of the specific antibodies of D 53 carried out by immunoelectrodifusion on 1% agarose gel, injected with *K. pneumoniae*

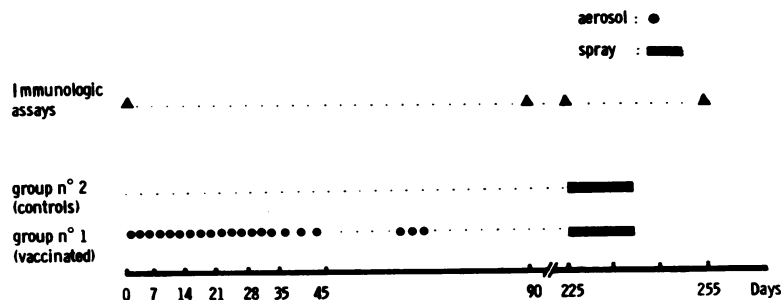


FIG. 1. Treatment and survey procedure.

antigens according to the technique of Laurell: 15 ml of 1% agarose gel was added to 150 μ l of *K. pneumoniae* antigen frequently controlled against a reference serum. The potential difference was 3 V/cm; the precipitates were dyed with Coomassie brilliant blue after washing and drying the plates. When the sera contained specific antibodies of *K. pneumoniae*, the height of the "rockets" of precipitates increased proportionally to the amount of these antibodies (Fig. 2).

RESULTS

Effectiveness. Effectiveness was measured by looking for an increase of specific antibodies (Fig. 3) as well as the possible correlation with the global immunoglobulin.

After the first series (day 90). Conventional statistical methods show that there are small time variations in total immunoglobulin, except the IgM, which increased in the treated subjects (Fig. 4). On the other hand, specific antibody increased distinctly in the vaccinated subjects. At the end of this conventional statistical study, it was important to know the relationships between the increase of the specific antibodies and the serum immunoglobulin

global levels. For this, we resorted to multifactorial analysis of the data.

The multifactorial analysis of the data shows first the harmonious distribution of the subjects with regard to the parameters adopted (levels of immunoglobulin, age, weight, and size) (Fig. 5).

Figure 6 shows an increase in the specific antibody in the majority of vaccinated subjects (except for 4 of them), whereas the levels in the control subjects remain mostly stable (42 times out of 53). Hence, a very clear distinction appears between the treated subjects and the controls, the latter having fairly constant global and specific antibody levels.

On the other hand, the treated subjects had variations in the global immunoglobulin levels concomitant with the appearance of specific antibody.

In fact, according to the importance in the variations, it is possible to distinguish two subgroups among the treated subjects: the first includes individuals who show a high increase in specific antibody. This goes together with the low initial levels of IgA and IgG, which increased in the course of the study, and the average levels

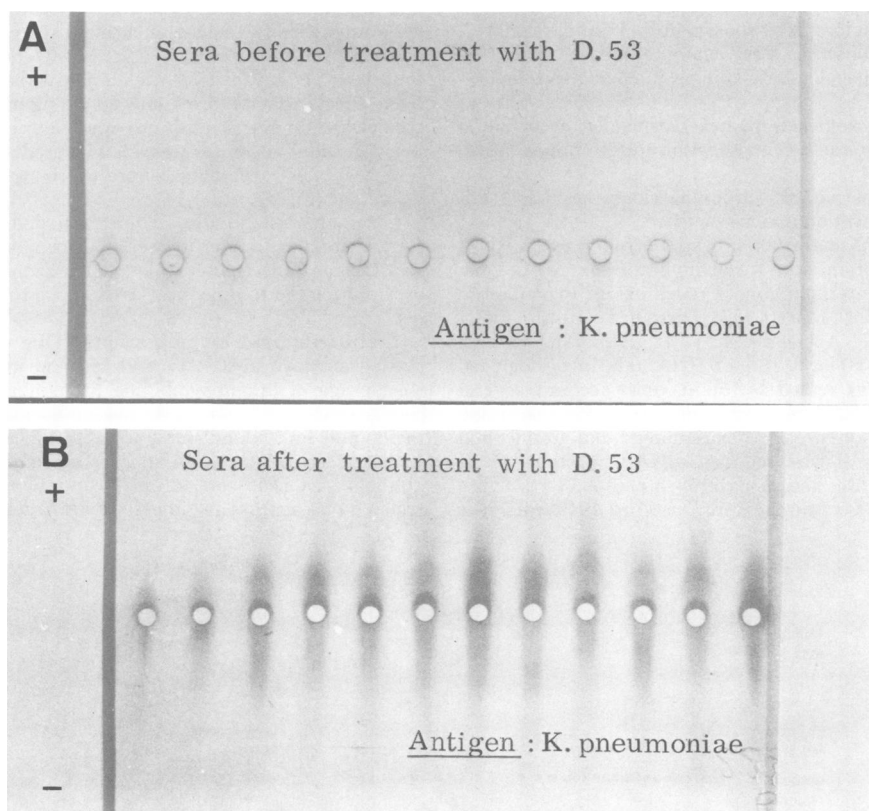


FIG. 2. Specific antibodies of D 53 (vaccine). (A) Serum of 12 subjects before treatment; (B) serum of 12 subjects after treatment.

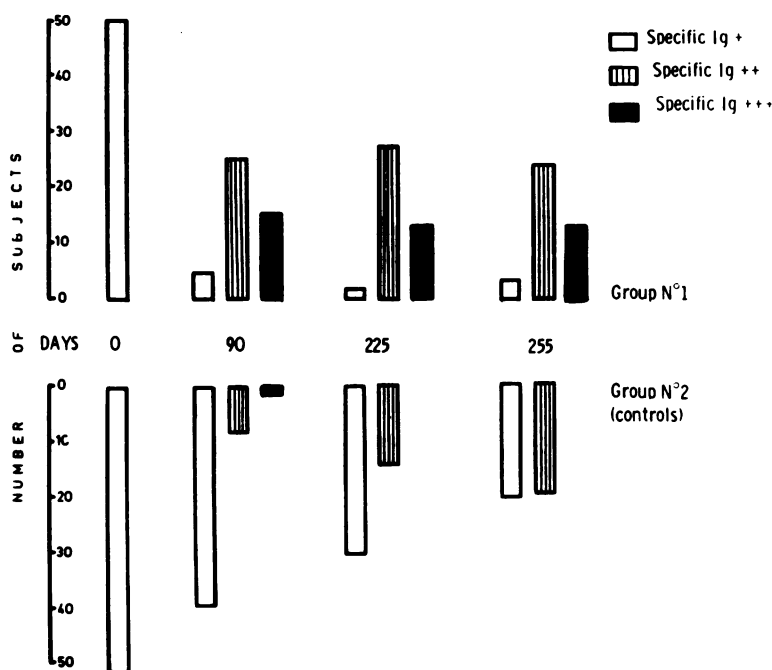


FIG. 3. Variations of specific immunoglobulins of *K. pneumoniae*.

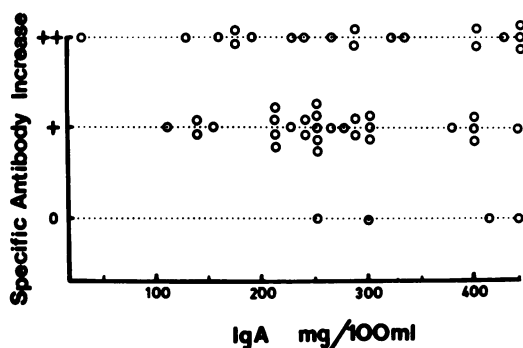


FIG. 4. Variation of specific antibodies according to serum IgA levels.

of IgM, which also increased. The second subgroup corresponds to a lower increase in specific antibody. Those have high IgA and IgG levels which remain stable with low IgM levels (Fig. 4).

Therefore, the production of specific antibody appears to go together with an IgM increase (Fig. 7).

Results of the second vaccination period (days 225 to 255). This experiment had a triple objective. The first objective was to estimate the long-term effects of the vaccine. After a period of 6 months, the specific antibody of the control group remained strictly stable in each case (52 subjects). Among the vaccinated subjects (47

subjects), the specific antibody levels mostly remained identical after the initial increase (36 subjects), 8 diminished slightly and only 1 increased (Table 1).

The second objective was to study the immunological behavior of the vaccinated subjects under the action of the booster. Among the individuals submitted to booster treatment, some did not show any new production of specific antibody and their IgG remained stable; others showed a new secretion of specific antibody and had a parallel increase in the IgG (Table 2).

The third objective was to investigate the immunological behavior of the "control" group (now treated) as compared with primo-vaccination of the "treated" group. The control group behaved more or less like the first treated group, with a parallel increase in the specific antibody and the IgG (Fig. 4).

Tolerance. The clinical tolerance of the supervision procedure was considered as excellent and did not require any interruption of treatment.

DISCUSSION

The results recorded in animals (Dussourd d'Hinterland et al., French patent Pierre Fabre S.A. 2.305.990, March, 1976; Dussourd d'Hinterland et al., B.O.P.I., Abstr. no. 49, p. 12, 179, 1976; Dussourd d'Hinterland et al., French

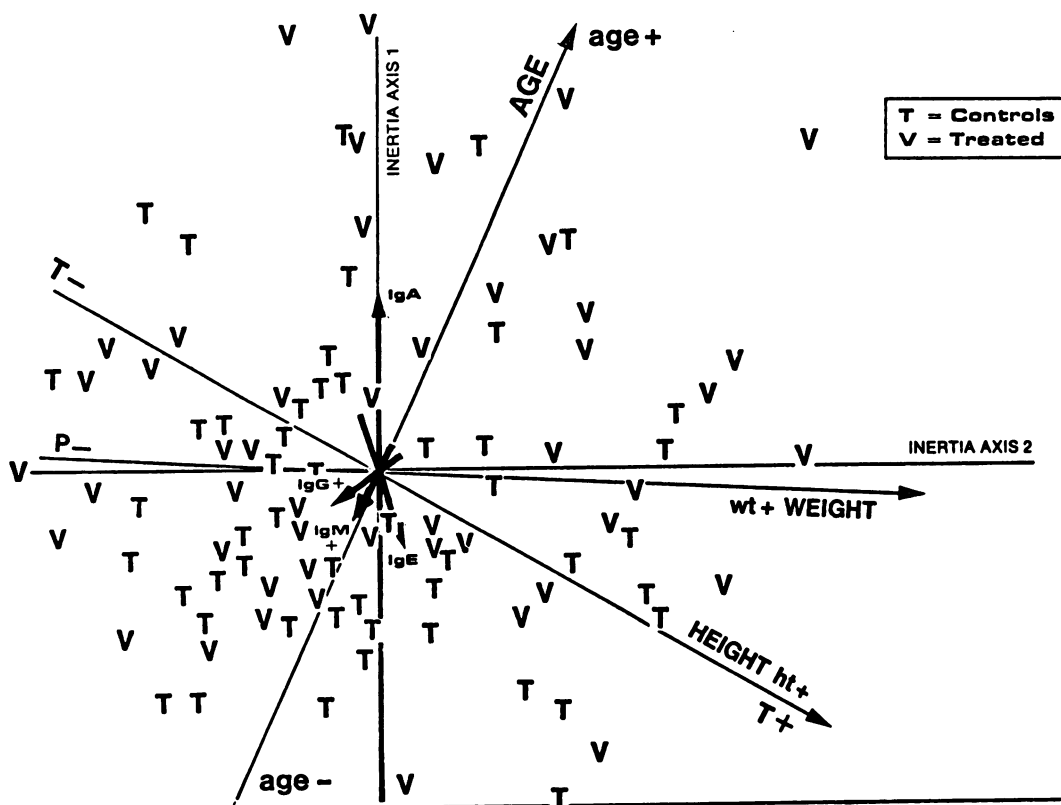


FIG. 5. Search for homogeneity among groups.

patent Pierre Fabre S.A. 2.253.499, August, 1976; Dussourd d'Hinterland et al., B.O.P.I., Abstr. No. 32, p. 9, 353, 1975) were confirmed in humans. There were definitely clear short-term results, and it should be stressed that the effectiveness of the immunization persisted long after vaccination. It is difficult to compare the effectiveness of D 53 with that of other types of antibacterial vaccines which have not been submitted to the same study.

Ganguly and Waldman (8) have stressed the individuality of the bronchopulmonary immunological system and compared local immunity with the systemic immunity. The secretory immune system (in which the IgA plays an important role) and the local cell-mediated immunity (17) are the first means of defense. Waldman and Henney (34) showed that immunization by the aerosol route presents many advantages as opposed to the parenteral route for the prevention of infection of the respiratory tract.

Although all the authors agree that this type of vaccine is strongly active, no fraction has yet been chemically isolated. Youmans and Youmans (44) have established that the isolated active fraction of the *Mycobacteria* is a double-

stranded RNA that is thermolabile, ribonuclease sensitive, and only active in the presence of an adjuvant. However, various results show that other subcellular fractions are concerned according partly to the methods and the strains used for the isolation of the ribosomal fractions, partly also to the bacteria concerned. Whereas Venneman and Berry (31) and Venneman and Bigley (32) isolated highly active RNA fractions at very low doses from *S. typhimurium*, Johnson (13, 14) thinks that the protective fraction is a ribosomal protein; Smith and Bigley (26) are rather in favor of an RNA-protein complex. According to Eisenstein (5) for *S. typhimurium*, the immunogenic activity of RNA of these germs is connected with the presence of O antigen. Working on *Francisella tularensis*, Andron and Eigelbach (3) think that RNA could play a structural or carrier role in the presentation of an unidentified somatic antigen which could be constituted by ribosomal proteins specific of this strain. A certain number of studies invoke ribosomal proteins as the principal immunogen in the cases of *N. meningitidis* (14), *S. aureus* and *Pseudomonas aeruginosa* (37, 38), *S. pyogenes* (24), or lastly *S. pneumoniae* (28). Jensen et al.

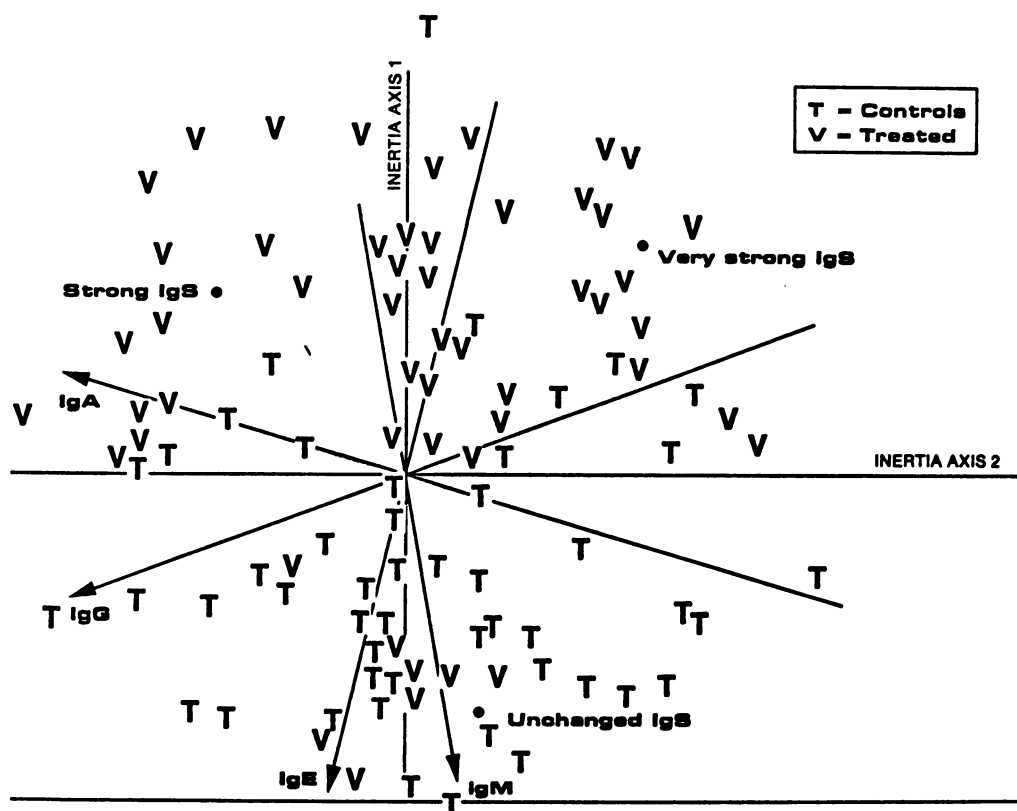


FIG. 6. Distribution of subjects according to immunoglobulin changes.

(12) feel that the protection against *V. cholerae* is due to the presence of a contaminant.

Concerning D 53, Dussourd d'Hinterland et al. (Arzneim. Forsch., in press) think that the RNA of the bacteria of this formula is the most important fraction. Immunizing laboratory animals (mice) with doses of 0.7 μ g of the purified RNA from *K. pneumoniae* and from *S. pneumoniae* (containing less than 0.5% of contaminants), these authors have obtained 100% vacinal protection, confirmed by the appearance of specific antibodies at levels six to eight times higher than those obtained with conventional microbial lysates.

The type of immune response induced depends not only on the pathogenic strain used, but also on the degree of purity of the ribosomes and of the RNA obtained. Roughly two types of immune responses are possible, according to the method of reproduction, intra- or extracellular, in vivo, of the bacteria concerned. Thus, according to Youmans and Youmans (40, 42, 43), the fractions prepared from bacteria with intracellular growth, like *M. tuberculosis*, induce an immunity with essentially cellular mediation, whereas opsonin antibodies are involved for bac-

teria with extracellular growth, like *S. pyogenes* (24) or *S. pneumoniae* (28). For optional intracellular growth species, like *S. typhimurium*, the ribosomal fractions induce a humoral (11) or cell-mediated immunity (26).

Lastly, all of the authors agree that the bacterial ribosomal fractions are effective. However, for ribosomal RNA (rRNA) the investigators are not all of the same opinion because the vaccine activity depends partly on the bacterial strain, partly on the vaccine purity, and partly, in some cases, on the formation of antigenic complexes with the other constituents of the bacterial cells. According to Youmans and Youmans (44), the rRNA of *M. tuberculosis* induces a specific immune response against an experimental tuberculosis, and these authors suggest that rRNA would be so powerful an activator of the reticuloendothelial system that the stimulated macrophages would become capable of phagocytizing the virulent cells more actively on contamination. It might consist of an increase in the nonspecific resistance.

Youmans thinks that the *M. tuberculosis* RNA would activate the immune system so that it responds more rapidly to a stimulation by an

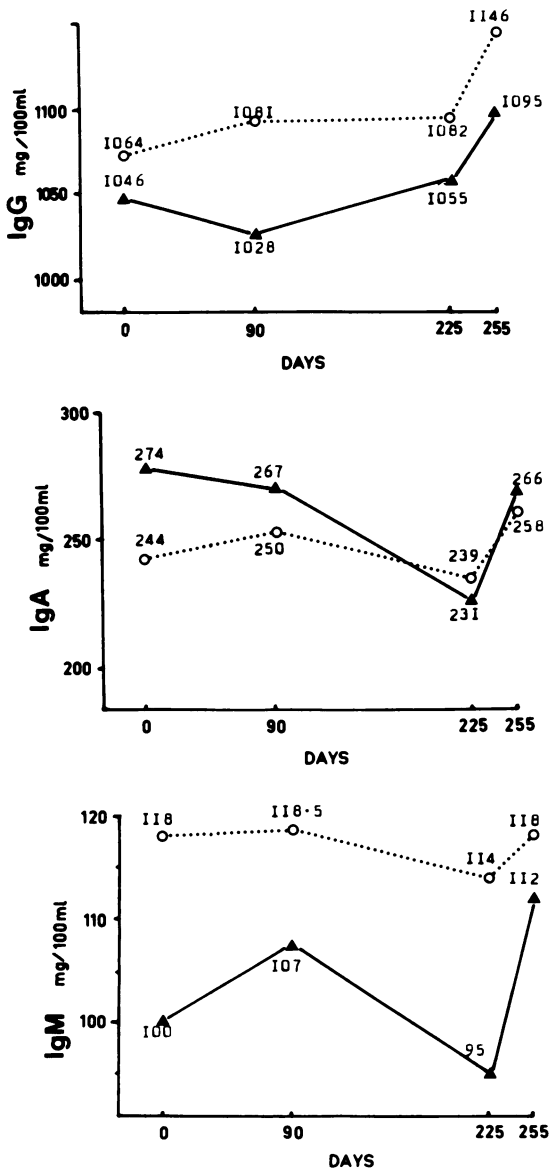


FIG. 7. Variation of immunoglobulins. Control subjects, group 2 (▲—▲); vaccinated subjects, group 1 (○····○).

antigen at a later time. Thus, the mycobacterial RNA would be, at the same time, a stimulant of the specific immune defenses and could, just like BCG, play the role of a powerful systemic adjuvant. On the other hand, the *Salmonella* RNA does not seem to be able to produce a specific immunity, except in the presence of small amount of polysaccharides. For D 53, Dussourd d'Hinterland et al. (Arzneim. Forsch., in press) think that, whereas for mycobacteria and strains

with intracellular parasitism, the immunogen is indeed rRNA and in particular RNA 23S—as Youmans and Youmans have illustrated—concerning bacteria composing the formula of D 53, experience seems to prove that the presence of slight traces of polysaccharides, coupled with the rRNA, is indispensable for the induction of a specific response.

However, these authors observe that if one associates polysaccharide antigen with purified RNA preparations, the immune response is not proportionally increased. These authors thought that for certain bacteria, rRNA coupled with traces of polysaccharides is very powerful. This structure could exist especially in ribosomes fixed to the cytoplasmic membrane, but we do not yet know if they are functional. It would form part of the antigenic fraction of the bacteria, or it would be formed during bacterial lysis due to the great affinity of RNA for some polysaccharides.

These hypotheses would explain the negative results found by Eisenstein (5) and Hoops et al. (10) with rRNA obtained from mutants of *S. typhimurium* free of O antigen. rRNA of these mutants is not more active or directed by the presence of polysaccharides. In fact, amounts of O antigen distinctly higher than those of rRNA must be administered to obtain an immune response. In their studies, Dussourd d'Hinterland et al. used doses of 0.7 to 1 μ g of rRNA containing less than 3.5 to 5 ng of contaminants (Dussourd d'Hinterland et al., *Arzneim. Forsch.*, in press). In these doses the polysaccharide antigens are inactive.

In most cases, immunization by ribosomal vaccines will induce an immunity to other strains of the same species. Thus, the ribosomal proteins of *S. pyogenes*, group A, type 14, protect mice against streptococci of type M 2, 5, 8, and 12 (24); the ribosomes of *S. pneumoniae* type 3 protect mice against types 1, 2, and 7 (30); or lastly the group B *N. meningitidis* ribosomes protect mice against serotypes A or C (29).

However, there do not seem to be crossed reactions between species. Thus, extracts of *S. aureus* do not protect against *F. tularensis* (3). Finally, the strains are more or less resistant: purified extracts obtained from the L VS (attenuated) strain of *F. tularensis* protect mice against the pathogenic strain 425 but not against SCHU 54 (3). For D 53, Dussourd d'Hinterland et al. (*Arzneim. Forsch.*, in press) have demonstrated crossed reactions between different types of pneumococci as well as for different types of streptococci, but they have noted the absence of reactions between species, thus confirming the work of previous authors.

TABLE 1. *Parameters and modalities—variation of specific antibody between days 90 and 225*

Subjects (total)	Variation of specific antibody ^a					Mean levels of specific antibody ^b (avg):			
	Drop of XX	Drop of X	Un-changed	Increase of X	Increase of XX	≤1	1.5	2	2.5
Treated (47)	6	16	20	4	1	2	9	22	11
Control (52)	2	9	36	5	0	36	14	2	0

^a X, Specific antibody titer +; XX, specific antibody titer ++.

^b A cross is counted as 1 unit.

TABLE 2. *Parameters and modalities—variation of specific antibody between days 225 and 255*

Subjects (total)	Specific antibody				Avg loss of specific antibody			
	Drop in production	Unchanged	Weak increase	Strong increase	≤1+	1.5+	2+	≥2.5+
Treated (45)	5	19	18	3	4	11	16	14
Control (51)	2	27	18	4	26	18	7	0

In animals, the majority of authors have used adjuvants, especially with *M. tuberculosis* (39) and *S. pneumoniae* (30). These authors used the Freund complete or incomplete adjuvant, medically unacceptable in humans owing to its toxicity. On the other hand, the adjuvants obtained from bacterial cell walls are effective and atoxic. In 1964 Shaw et al. (25) compared the effects of adjuvants from various bacterial strains with the effects of mycobacteria and concluded that the latter were much more effective due to the particular chemical structure of their cell wall.

In 1972 three different groups (2, 9, 18) showed that the soluble substances obtained from mycobacteria were as active as the whole bacteria, the bacterial cell wall, or the Freund complete adjuvant (which contains a suspension of killed mycobacteria emulsified in mineral oil). Ellouz et al. (6) demonstrated that the adjuvant activity was a general property of peptidoglycans of the walls of various bacterial species, whatever their origin or Gram-stain reaction.

The immune effects of the final fractions are not always identical, because the majority of authors note a mixed effect in the induction of delayed hypersensitivity and the production of antibodies, whereas Nauciel et al. (22) only record the former.

In spite of these differences and perhaps essentially attributable to the investigation procedures, it is undeniable that the peptidoglycans of the bacterial walls have an adjuvant effect on humoral and cellular immunity, whatever the origin of the bacterial strain.

In animals, Nakashima et al. (20, 21) showed the potent properties of membranal and capsular extracts of *Klebsiella* on the in vivo and in vitro immune response; these preparations, however, contain lipopolysaccharides (15), which are another bacterial adjuvant that has been known

for a long time but which are toxic. Lefevre and Agneray (16) have studied the immunostimulant effect of an extract of *K. pneumoniae* devoid of lipopolysaccharide and antigenicity in rabbits or guinea pigs. However, this fraction stimulates the development of delayed hypersensitivity in guinea pigs and that of the humoral immunity of rabbits, and lastly in C57Bl/6 mice produces an immunostimulation lasting 3 weeks which can be revealed by a primary or secondary response. White and Maguire (36) think that as far as their operating system is concerned, the peptidoglycan monomeric or polymeric derivatives are active on cellular or humoral immunity and increase the activity of macrophages. Usually, it is not necessary to incorporate them in oil. They are active in aqueous solution.

Thus, the glycopeptide compound soluble in water has an adjuvant effect in vivo (2) and in vitro makes mixed cultures of lymphocytes (4) and the formation of antibodies (19) possible. However, the glycopeptide has no B mitogenic action; neither has it any part in the liberation of macrophage factors. These functions belong to the glycodipidic compound of mycobacterial cell walls (23). The peptidoglycans are atoxic (27) in the various experimental animals used.

ACKNOWLEDGMENT

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