THE EXPERIMENTAL PRODUCTION OF VACCINE STRAINS OF SHIGELLA FLEXNERI AND THE STUDY OF THE IMMUNOLOGICAL EFFECTIVENESS OF A LIVING DYSENTERY VACCINE

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The problem of obtaining living vaccines for the specific prophylaxis of enteric infections continues to be one of urgent importance. Great importance in this respect is attached to work on the development of a living dysentery vaccine [16, 19].

In the present paper we describe the results of four years' (1954-57) experimental research on the production of vaccine strains of Shigella flexneri and on the investigation of the properties of the living dysentery vaccine [4, 5].

TABLE 1

The Principal Biological Properties of the Test Strains

Serial No.	Identification No. of strain of Shigella flexneri	Whence isolated	Date of isolation	Virulence LD^{50}(in 10^6)	Antigenicity in %	Toxicity, LD^{50}(in 10^9)	Titer of agglutination with specific serum
1	No. 6267, type c	From stools	10/22/52	250	86	4	1:12 800+++
2	No. 6211, type f	"	10/17/53	250	75	4	1:12 800+++
3	No. 266, type a	"	2/15/50	250	80	2	1:12 800+++

EXPERIMENTAL METHOD AND RESULTS

Three strains of Shigella flexneri — No. 6267 type c, No. 6211 type f and No. 266 type a — were used in the experiments. The first two were obtained from the patients with acute dysentery and the last was a production strain obtained from the government control institute. Our aim was to obtain a considerable and lasting fall in the virulence of these strains while preserving their antigenicity and other biological properties.

For this purpose we carried out experiments on the cultivation of Shigella flexneri in bile in conjunction with passage through cold-blooded animals insusceptible to dysentery. Bile is known to stimulate the growth of a number of microorganisms and to stabilize their newly acquired properties. Repeated subculture in bile broth

TABLE 2. Characteristics of the Principal Properties after 10 Passages through the Frog's Brain

Serial no.	Strain no.	Morphological and staining properties	Biochemical properties	Form of dissociation	Titer of agglutination	Virulence (in 10^9)	Antigenicity (in %)
1	6267	Typical	Typical	S	1 : 12 800	5	86
2	6211	"	"	»	1 : 12 800	1	65
3	266	"	"	»	1 : 12 800	1	80

of the microorganisms of anthrax, swine erysipelas and other diseases brings about a sharp reduction in their virulence and stable preservation of newly acquired properties [17]. When Shingella flexneri is grown in 20% bile broth, a sharp decrease in its virulence is observed [10].

The use of cold-blooded animals for the experimental cultivagion of dysentery microorganisms was established long ago. In 1884, I. I. Mechnikov observed that the virulence of Bacillus anthracis was greatly diminished by injection under the skin of frogs. When toads (Bufo vulgaris) were inoculated with Bacillus pestis, it was found [3] that the longer the organism existed in the body of the cold-blooded animals, the greater was the decrease in its virulence. Similar experiments with Bacterium tularense also confirmed the possibility of lowering the virulence of these microorganisms [12].

The dysentery strains tested all possessed characteristic biological signs (Table 1).

All the properties were assessed by the generallly accepted method. The virulence and toxicity were estimated by the value of the LD_{50}, the results obtained being treated by the method of Read and Mench.

It will be seen from Table 1 that the strains possessed high virulence and antigenicity and low toxicity and were agglutinated to a titer of 1:12 800. Also, they were present in the S-form, they possessed high specificity and were phagolytic.

In the first series of experiments the optimal concentration of bile-peptone broth and cultivation time were selected. As a result several important factors were elucidated. The greatest fall in virulence took place during cultivation at constant temperature (37°) in 60% bile-peptone broth. From the LD_{50} values, the virulence of strain No. 6267 was 5 x 10^9/ml, and that of strains Nos. 6211 and 266 was 750 x 10^6/ml. The antigenicity remained high (between 60 and 80%), even after cultivation for 120 days in 60% bile-peptone broth. The remaining biological properties, namely agglutinability, specificity, form of dissociation, saccharolytic activity, etc. remained unchanged. It was remarkable that the cultivation of Shigella flexneri in bile-broth brought about a decrease in virulence but usually stabilized all the remaining properties typical of this species of microoganism.

However, the level to which the virulence of strains Nos. 6211 and 266 had been lowered was still too high, and furthermore, after passage through white mice, the virulence reappeared. We therefore decided to cultivate the microorganisms in bile-peptone broth and to combine this with cultivation in cold-blooded animals. These experiments were performed on pond frogs (Rana radibunda), which were inoculated intracerebrally. From our point of view, this method of introduction of the infective material had two advantages: firstly, the usual medium for its existence in an insusceptible animals must promote a further lowering of the virulence of the microorganisms, and secondly, it would be easier to obtain a pure culture, uncontaminated by other microorganisms, from the frog's brain.

The culture was injected into the midbrain in the region of the posterior third of the parietal bones. A 24-hour culture, suspended in physiological saline, containing 10^9 bacterial cells per ml was used for inoculation. The volume of the suspension injected was 0.05 ml. For passage, 48 hours after inoculation, the frog's skull was opened, the brain extracted and seeded with a pure dysentery culture which, after thorough testing, was again injected into the brain of a frog. Ten passages were performed.

Table 2 shows the results of tests of the strains after the last passage, indicating that they preserved their

TABLE 3

Changes in the Virulence and Antigenicity after Cultivation in a Medium Containing Bile and Passage through the Frog's Brain

Serial no.	Strain no.	1st passage				2nd passage				3rd passage			
		60% bile broth		frog's brain		60% bile broth		frog's brain		60% bile broth		frog's brain	
		virulence LD_{50} (in 10^9)	antigenicity (in %)	virulence LD_{50} (in 10^9)	antigenicity (in %)	virulence LD_{50} (in 10^9)	antigenicity (in %)	virulence LD_{50} (in 10^9)	antigenicity (in %)	virulence LD_{50} (in 10^9)	antigenicity (in %)	virulence LD_{50} (in 10^9)	antigenicity (in %)
1	6267	5,0	80	5	80	5	80	5	89	5	87	5,5	85
2	6211	4,5	73	5	70	4	70	4	79	4	76	5,0	75
3	266	5,0	71	5	71	4	73	4	88	5	76	5,0	78

TABLE 4

Changes in the Virulence and Antigenic Properties of "Reversion" Strains of Shigella flexneri

Serial no.	Strain no.	Total number of subcultures	Duration of subcultures	1955		1956				1957			
				Quarters									
				III	IV	I	II	III	IV	I	II	III	IV
1	6267	654	2 1/2 years	$\frac{4\times10^9}{80\%}$	$\frac{5\times10^9}{84\%}$	$\frac{5\times10^9}{88\%}$	$\frac{4,5\times10^9}{86\%}$	$\frac{4\times10^9}{85\%}$	$\frac{5\times10^9}{86\%}$	$\frac{5\times10^9}{85\%}$	$\frac{4\times10^9}{84\%}$	$\frac{4\times10^9}{85\%}$	$\frac{3,5\times10^9}{80\%}$
2	6211	304	1 year						$\frac{3\times10^9}{75\%}$	$\frac{3\times10^9}{72\%}$	$\frac{2\times10^9}{70\%}$	$\frac{2\times10^9}{75\%}$	$\frac{3\times10^9}{70\%}$
3	266	654	2 1/2 years	$\frac{4\times10^9}{82\%}$	$\frac{4\times10^9}{80\%}$	$\frac{4\times10^9}{88\%}$	$\frac{4\times10^9}{75\%}$	$\frac{4\times10^9}{82\%}$	$\frac{3\times10^9}{70\%}$	$\frac{3\times10^9}{72\%}$	$\frac{4\times10^9}{77\%}$	$\frac{3\times10^9}{80\%}$	$\frac{3\times10^9}{75\%}$

Note. Legend: numerator — virulence as shown by the value of LD_{50}; denominator — antigenicity by survival rate of white mice.

TABLE 5. Immunobiological Changes in Kittens after Immunization and Infection (Mean Values)

Serial no.	Description of material	Agglutination reaction				Phagocytic reaction			
		before experiment	after vaccination			before experiment	after vaccination		
			9th day	14th day	22nd day		9th day	14th day	22nd day
1	Living vaccine	Negative	1:400	1:400	1:200	9 11	24 31	30 37	27 36
2	Heat-treated	The same	1:62	1:62	1:50	10 12	20 25	22 24	16 21
3	Mixture of virulent initial strains	Negative	1:270	1:220	1:200	7 10	27 31	32 36	33 40

Note. Legend: numerator — specific phagocytic reaction; denominator — nonspecific phagocytic reaction.)

TABLE 6. Properties of Living Dysentery Vaccine at Different Periods of Storage

Strains composing the vaccine	Original properties			1st quarter			2nd quarter			3rd quarter		
	virulence LD_{50} (in 10^9)	antigenicity (in %)	toxicity LD_{50} (in 10^9)	virulence LD_{50} (in 10^9)	antigenicity (in %)	toxicity LD_{50} (in 10^9)	virulence LD_{50} (in 10^9)	antigenicity (in %)	toxicity LD_{50} (in 10^9)	virulence LD_{50} (in 10^9)	antigenicity (in %)	toxicity LD_{50} (in 10^9)
6267, type c 6211, type f 266, type a	5	87	4	5	85	4	4	80	3,7	3	80	3,5

principal typical properties, and primarily their antigenicity, although the values of the LD_{50} for strains 6211 and 266, a measure of their degree of virulence, still remained in the region of 10^9. In order to obtain a greater lowering of virulence we studied the possible periods of keeping the culture in the frog's brain. All these strains were kept in the brain for 20, 25, 30, 40 and 45 days.

After 20 days the virulence of strain 266 showed a considerable fall, as shown by the value of $LD_{50} - 5 \times 10^9$. After 30 days the virulence of strain 6211 also reached these figures. So far as the effect of the period of keeping on the properties of the strains is concerned, they still divided actively after 30 days, and preserved their principal typical properties. At still later periods, some dying away of the microorganisms took place, although they continued to divide.

In the next series we undertook serial cultivation of strains in bile-peptone broth and frog's brain. We named each passage in turn, and tested three such passages. In each passage, cultivation was in 60% bile-peptone broth for 30 days; in the first passage the culture was kept in the frog's brain for 45 days and in later passages 30 days. After each passage complete testing of the culture was carried out.

The results in Table 3 reveal usually stable indices of virulence and antigenicity in the course of the passages. According to these indices, all three strains could be described as highly antigenic and with virulence equivalent to an LD_{50} value of 4 and 5×10^9. Full testing of the remaining biological properties satisfied us that they preserved the typical signs of this species of microorganism.

Systematic experiments in possible reversion of virulence were conducted by seeding all three strains on solid and liquid nutrient media.

Reversion of virulence experiments on strain 6267 were started in August and those on strain 266 in September 1955, those on strain 6211 in September 1956. The cultures were seeded on Petri dishes with weakly alkaline agar. After 18-20 hours S-form colonies were picked off the dishes, checked by the trypaflavine reaction and subcultured on agar slopes in test tubes. The biological properties of the 24-hour cultures on agar slopes were studied. We know from the literature that frequent subcultures of Shigella flexneri usually react in dissociation to R-forms and to loss not only of virulence but of antigenic properties also [7, 9, 13].

Virulence and antigenicity trails of the serial subcultures of the reversion strains demonstrated the usual stability of these properties (Table 4). All three strains retained their typical morphology, were agglutinated by specific serum to a limiting titer (1:12 800), were present in the S-form of dissociation, retained their typical biochemical activity and were not agglutinated by heterogenic sera, i.e., they remained specific.

The next series of experiments was devoted to the production of a living dysentery vaccine from the vaccine strains studied, and to the testing of its properties. Several series of vaccine were prepared and two were studied in detail: the first, prepared in December 1955, was from two strains – 6267 and 266, and the second, prepared in December 1956, was from three strains – 6267, 6211 and 266. The vaccine was prepared by washing a 24-hour culture of the microorganisms with physiological saline. A standard for the suspension was established at 4×10^9/ml. The usual method of quality control were employed. The vaccine selected for the experiment had the typical morphology and antigenicity, low toxicity and virulence equivalent to an LD_{50} value of 4 x to 5 x 10^9. The antigenic properties of the vaccine were further determined in experiments on kittens. A large volume of information has now been accumulated on the possibility of reproducing dysentery infection in kittens and adult animals [1, 2, 11, 14, 15, 18].

In our experiments 15 kittens were used in trails of the vaccine prepared from two strains (6267 and 266). Natural infection of the animals with the Shigella and Salmonella groups of microorganisms was first excluded, as also was the presence of immunity. The animals were divided into three groups: the 1st group (6 kittens) was immunized with living dysentery vaccine, the 2nd group (4 kittens) – with heat-treated vaccine (prepared from the original strains and killed by heating) and the 3rd group (5 kittens) received the same dose of a mixture of the original virulent strains. The animals were immunized and infected once only, by mouth, after being starved for 24 hours, being given 4×10^9 bacterial cells suspended in 2 ml of warm milk.

The experiments showed that the control group of kittens became ill 4-6 days after infection, with characteristic clinical manifestations – dyspeptic disorders, raised temperature, adynamia and other signs [3, 4]. No clinical manifestations of the disease were observed in the kittens receiving living and heat-treatment vaccines.

The immunobiological changes as a result of immunization and infection were tested with serum from the experimental animals by means of the agglutination and phagocytic reactions. The phagocytic activity of the leucocytes was studied. So that the trend of these changes could be followed, the rezctions were carried out on different days after administration of the material.

The results in Table 5 show that such a weak immunizing stimulus as a single oral immunization with living vaccine caused a marked accumulation of antibodies on the 9th-14th day after administration of the material. The antibody titer after immunization with heat-treated vaccine lagged behind considerably. Similar features could be observed on analysis of the trend of both the specific and the nonspecific opsonophagocytic reaction.

Experiments on vaccination of kittens with living dysentery vaccine were repeated in 1957. Twenty-four animals were used, divided into two equal groups: one of these was vaccinated and the second infected with a mixture of original virulent strains. The preparation tested was a living vaccine prepared from three strains (6267, 6211 and 266) in December 1956.

Immunobiological changes in the two groups of animals were tested alone the same lines. As additional measures, the complement fixation reactions was carried out with serum from the immunized animals, and the hapten reaction with the animals' feces. The most delicate and earliest test was the complement fixation reaction, which was positive only 48 hours after vaccination. Diagnostic titers in the agglutination reaction were observed on the 6th day, and at the same time the hapten reaction also became positive. A marked increase in

the phagocyte activity of the leucocytes was observed after 72 hours, and this increased progressively in the subsequent tests. The immunobiological changes in the immunized kittens were no less pronounced than those in the infected animals.

The properties of the last vaccine were tested at intervals during storage in the refrigerator for 9 months.

The results in Table 6 show that during the period of observation the vaccine remained avirulent, nontoxic, and highly antigenic. Monthly testing of the survival of the vaccine, carried out by the method used for assay of living plague vaccine, showed that it varied between limits of 50% (after storage for 1 month) and 30% (Storage for 9 months).

SUMMARY

As a result of observations conducted for many years, the authors have developed a method of obtaining avirulent and high immunogenic strains of Shigella flexneri. The principle of this method consists of cultivating the microorganisms in cold-blooded animals (pond frogs) resistant to this infection, as well as on animal products (60% bile-peptone broth).

An usual stability of the acquired properties, which is not characteristic for this species of bacteria, was established. 3 experimental strains appeared to be avirulent, and highly immunogenic and retained all the biological properties. The possibility of studying dysentery immunization problems on young cats was demonstrated.

Living Shigella flexneri vaccine possesses immunological efficacy in experiments on this species of animals; it may be stored in a refrigerator up to 9 months without losing its properties.

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