axils, colocasia, certain bananas, tree holes, coral holes, and snail shells, in order of preference.

Ae. simpsoni is also known to breed frequently in man-made containers in South Africa (Muspratt, 1956). Wiseman et al. (1939) in Kenya reported it breeding in many man-made containers, pools and puddles, rivers and streams, fallen leaves, coconut shells, snail shells, etc. Lewis (1943) even found *Ae. simpsoni* breeding in a rock pool in granite in the Nuba Mountains of Sudan.

The coral rock holes on the Msasani peninsula near Dar es Salaam, Tanzania, are considered to be newly-recorded breeding sites of *Ae. simpsoni*.

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Preliminary Studies on the Development of a Gonococcal Vaccine *

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As it now seems unlikely that gonorrhoea can be contained by chemotherapeutic agents, there appears to be some need for a vaccine for prophylaxis. The development in recent years of better media and better culture techniques has considerably improved the chances of developing such a vaccine. In the present report, we describe in detail the production of a somatic antigen vaccine and the results of a small trial on human volunteers in which the effectiveness of the vaccine was assessed by the measurement of two types of antibodies—one determined by bentonite flocculation and the other by a tissue culture inhibition test.

Materials and methods

Bacterial strains. All the strains used in the study were freshly isolated strains received on chocolate agar slants from the Ontario Public Health Laboratories, Toronto. Upon receipt, the cultures were streaked on plates with Columbia Blood Agar Base (CBAB) (BBL) and type I colonies (Kellogg et al., 1963, 1968) were picked and again cultured on CBAB plates; they were incubated for 24 hours at 37°C and the resulting growth was harvested in 2% skim milk (Difco) and then lyophilized. All cultures were incubated in an atmosphere containing 10% CO_2. Two strains identified as Canadian Communicable Disease Centre (CCDC) No. 172 and 173 were used for vaccine and CCDC No. 138 was used in the tissue culture antibody inhibition tests.

Culture medium. For vaccine production, *Neisseria* chemical defined medium (NCDM) [5] described elsewhere (Kenny et al., 1967) was used.

Vaccine production. Tubes of the lyophilized vaccine strains were rehydrated in NCDM and plated on CBAB plates and incubated overnight in an atmosphere containing 10% CO_2. The inoculum was

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[5] Obtained in powder form from the Grand Island Biological Company, Grand Island, N.Y., USA.

prepared by harvesting the resulting growth in NCDM and preparing a homogeneous suspension in this same medium. The inoculum was adjusted in NCDM to a count (as determined by a model " B " Coulter Counter) of 1×10^6 organisms per ml and then 1.0 ml was inoculated into individual Erlenmeyer flasks, each of which contained 300 ml of NCDM. The flasks were then incubated for 24 hours in an atmosphere containing 10% CO_2 in a New Brunswick Controlled Environmental Incubator Shaker, the shaker being set to rotate 120 times per minute. The bacterial count at the end of this period averaged 2.5×10^7 organisms per ml. The organisms were then killed by adding thiomersal to a final concentration of 1 : 10 000, and the flasks were left at room temperature until a 70% autolysis occurred (as judged by Coulter Counter determinations). The vaccine was then pooled and tested for sterility, for safety (using mice and guinea-pigs), and for antigenicity[1] (using rabbits). If these tests were satisfactory, the vaccine was cooled to 4°C and placed in vials. All tests for sterility, safety, and antigenicity were repeated on the finished labelled vials.

Antibody tests

(1) The bentonite flocculation test (Wallace et al., 1970) was carried out as summarized briefly below: 10 ml of stock bentonite were centrifuged for 10 min at 2 800*g*, the supernatant fluid was discarded, and the pellet was suspended in 1 ml of distilled water. To this suspension were added 2 ml of Sorensen's buffer, pH 6.2 (0.06M), 2 ml of gonococcus antigen, prepared as described in the above reference, and 2 drops of normal rabbit serum. After shaking, the mixture was incubated for 1 hour in a water bath at 37°C. The volume was then brought to 15 ml with distilled water and the suspension was centrifuged for 10 min at 2 800*g*. The supernatant fluid was discarded and the pellet was suspended in 5 ml of distilled water and 0.5 ml of 1 : 10 000 methylene blue (aqueous) was added. The mixture was well shaken and left for 3 min; the volume was again brought to 15 ml with distilled water and the mixture was again centrifuged. The pellet was washed twice more with 10 ml of distilled water and the particles were suspended in Sorensen's buffer, pH 7.2 (0.06 M). This was the final antigen and no further stabilizers were necessary.

For the actual test, serial dilutions of serum in 0.1 ml of saline were made in disposable cavity slides and 2 drops (0.1 ml) of sensitized bentonite were added to each cavity. The slide was shaken for 20 min (100 rotations/min) on an Eberbach rotator[2] and read immediately at low magnification (×60). The final dilution of serum that clumped all the particles was taken as the antibody titre.

(2) The tissue culture inhibition test (Diena et al., 1970) was carried out as described below:

(*a*) The cell culture (RE2) used was established in 1965 in the Virus Laboratories of the Canadian Communicable Disease Centre from trypsinized primary monkey kidney cells obtained from a female rhesus monkey. The cells are cultured routinely in Roux bottles containing M 199 medium (Morgan et al., 1950) with 10% fetal calf serum and 1% glutamine. Following trypsinization, the monolayers were dispersed and 2 ml aliquots of RE2 suspension (approximately 2.6×10^4 cells/ml) were dispensed in tissue-culture tubes, which were then incubated at 37°C in an upright position.

(*b*) To prepare the bacterial inoculum *Neisseria gonorrhoeae*, strain 138, was grown overnight on CBAB plates. The growth was scraped from the plates and suspended in saline to an opacity equivalent to that of 10 International Opacity Units (1×10^9 cells/ml). Dilutions were then made to $10^{-6.3}$ in Hanks' balanced salt solution (containing the same additive as the NCDM described by Kenny et al., 1967). This dilution, which was used for the inoculum, was equivalent to 100 LD_{50} for our tissue culture.

(*c*) To carry out the tissue culture inhibition test 1-ml aliquots of bacterial inoculum, prepared as above ($10^{-6.3}$), were mixed with 1-ml aliquots of serum dilutions (1 : 10, 1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320) and incubated at 37°C for 30 min. Four drops of the serum–bacteria mixture (0.2 ml) were added to each of 3 tubes of RE2 tissue culture, and incubated at 37°C for 40 hours. At the end of the incubation period, the fluid was removed from each tube and replaced with 2 ml of agar overlay (see below). The tubes were reincubated at 37°C for 72 hours.

(*d*) The agar overlay medium was prepared by mixing equal volumes of 3% agar and double

[1] Antigenicity was established by the presence of " inhibiting antibodies " developed in rabbits following the intravenous injection of the test vaccine.

[2] Eberbach Corporation, Ann Arbor, Michigan, USA.

strength M199 medium containing 5% calf serum, 0.2% skim milk, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and neutral red at a final concentration of 1 : 30 000—but no phenol red.

(*e*) The reading of the tissue culture tubes was facilitated by the fact that where the tissue cells remain viable, they retain the neutral red at the bottom of the tubes and appear red, while the agar layer remains clear. Where tissue culture destruction has taken place, the monolayer fails to take up neutral red and the agar layer becomes yellowish-brown.

The final dilution of serum that prevented the destruction of the tissue cultures by the gonococci was taken as the titre of that serum.

Immunization and results

The human subjects in the study were volunteers from our own department and science students attending the University of Ottawa. Careful histories were taken of each individual, paying particular attention to any history of allergies or of gonorrhoea. Persons with a history of allergies were not accepted. None of the subjects had a history of gonorrhoea. Each subject was interviewed following each immunization and careful notes were taken of reactions. With one exception (discussed later), the only reaction reported was pain at the site of the injection, which sometimes persisted for 24 hours. The pain was greatest after the first injection and was very much less after the second and third injections. In general, the reactions to the vaccine were quite mild.

Each individual was given three intramuscular injections of 1.0 ml at intervals of 3 weeks. Blood samples of approximately 10 ml were taken immediately before the first injection and 10 days after the third. The sera were separated and coded and their identity was not disclosed until all the titrations had been completed. All sera were held in the frozen state in liquid nitrogen (−195.8°C) until tested.

A total of 54 persons (17 female and 37 male) completed the study. The age and sex of the participants and the results of antibody titrations of both the pre- and the post-immunization antisera are shown in Table 1: 40 (74%) were in the 20–40-year age group—the group shown to have the highest incidence of reported gonorrhoea in Canada (Davies, 1969).

The significance of the bentonite flocculation antibody is not known. There is, however, no evidence to associate it with protection even though it would appear to be a useful adjunct for the detection and confirmation of gonorrhoea. The reciprocal of the flocculation titre was 4 or higher in 11 (20%) and was 1 024 in 1 (No. 11) of the 54 pre-immunization sera. With the exception of subject No. 11, whose titre remained the same following immunization, all subjects with positive titres in their pre-inoculation sera had at least a two-fold rise in titre in their post-immunization sera. Of the 43 with no pre-immunization titre, 31 (72%) developed flocculating antibodies following immunization and 12 (28%) showed no rise in these antibodies.

The tissue culture inhibition test detects the presence of inhibiting antibodies—bactericidal, bacteriostatic, or both—and while there is no proof that such antibodies are synonymous with protection, there is every reason to assume that they may play some part in an individual's resistance to the disease. The development of this type of antibody following immunization could, therefore, be highly significant. Altogether 18 (33%) of the pre-immunization sera were found to have inhibiting antibodies—12 having reciprocal titres of 10, 5 titres of 20, and one a titre of 40. The absence of a past history of gonorrhoea in any of the subjects suggests that these antibodies were not entirely specific for *N. gonorrhoeae*. Following immunization, however, every vaccinated subject with pre-immunization inhibiting antibody was found to have an increase in this antibody—13 had 8-fold rises, 4 had 4-fold rises, and 1 had a 2-fold rise.

Of the 36 subjects with no pre-immunization titre, 32 (89%) developed inhibiting antibodies following immunization, all but 3 having reciprocal titres of 40 or higher. Four persons (11%) failed to develop any measurable antibodies. Each of these, however, showed a good flocculating antibody response to vaccination. At the conclusion of the study, 47 (87%) had titres of inhibiting antibody of 40 or more.

DISCUSSION

The continuing high incidence of clinical gonorrhoea, as demonstrated by vital statistics (Davies, 1969), has shown clearly that neither penicillin nor any other chemotherapeutic agent is ever likely to contain the disease. There is a constant search for new and more effective therapeutic agents, but other methods will have to be used before satisfactory control of this disease can be achieved. A reasonable step to this end would be prophylactic vaccination with an effective vaccine. The need for such a vaccine

Table 1. Antibody titres before and after vaccination with gonococcal vaccine

No.	Age	Sex	Bentonite flocculation titre [a]		Tissue culture titre	
			Pre-immunization	Post-immunization	Pre-immunization	Post-immunization
2	26	F	0	128	0	0
3	48	F	0	0	0	80
4	47	F	16 (8)	128	0	0
5	46	M	0	32	0	40
7	32	F	0	0	0	80
8	26	M	0	8	0	80
9	63	M	0	256	0	40
10	32	M	0	64	0	20
11	48	M	1024 [b]	1024	0	80
13	47	M	0	32	0	80
14	33	M	0	16	40	160
15	42	M	8	16	0	40
16	40	M	0	0	0	80
17	41	M	0	32	0	80
18	30	M	0	16	0	40
19	37	F	128	256	0	80
20	23	M	0	8	0	80
21	28	M	4	32	0	10
22	27	M	0	128	0	0
23	25	M	16	512	0	0
24	20	F	0	128	0	80
25	20	M	128	512	0	40
26	21	M	0	256	0	40
27	21	M	0	32	0	80
28	21	F	0	64	0	40
29	22	M	0	32	20	80
30	26	M	0	16	0	40
31	22	M	32	64	20	160
32	23	M	0	0	0	40
33	22	M	0	0	10	80
34	20	F	8	128	0	80
35	23	F	0	0	20	80
36	20	F	0	0	0	40
37	19	F	0	512	10	80
38	21	M	64	128	10	80
39	20	M	0	16	not done	80
40	22	M	0	0	10	20
41	63	F	0	32	10	160
42	31	M	0	0	0	80
43	53	F	4	64	0	80
44	30	F	0	32	10	80
45	55	M	0	32	10	80
46	53	M	256	512	20	160
47	22	F	0	0	0	80
48	24	M	0	0	0	160
49	53	M	0	256	10	80
50	23	F	0	0	20	160
51	28	M	0	32	0	80
53	28	M	0	256	10	80
54	31	M	0	32	10	80
55	21	F	0	32	10	80
56	20	M	0	16	0	40
57	52	M	0	1024	10	40
58	35	M	128	512	0	40

[a] Titre is expressed as the reciprocal of the serum dilution.

[b] Since this volunteer reacted strongly to the second injection, he did not receive a third injection.

was recognized by the WHO Expert Committee on Gonococcal Infections (1963), which stated that " there was an urgent need to pursue research aimed at the development of at least a partially protective immunizing agent."

To the best of our knowledge, gonococcal vaccines are not in use today for either the treatment or the prevention of gonorrhoea but vaccines were used extensively up to the mid-1930s Tulloch (1929).

As a result of the earlier experience it was felt, in general, that there was little convincing evidence that the vaccines were of value in treating the acute phase of the disease since most cases cleared whether vaccines (or other treatment) were used or not. Most physicians, however, reported favourably (Tulloch, 1929) on the value of vaccines in the treatment of chronic cases and of complications. The reason given for this is, perhaps, of significance. It was felt that the vaccines established a basal general immunity, which prevented the disease from spreading in the host.

With the therapeutic agents now available there is little justification for using vaccines in therapy. The need today is for a vaccine for prevention and it is to this end that our efforts are being directed. As pointed out by the WHO Expert Committee on Gonococcal Infections (1963), a vaccine that affords even a partial protection or an immunity of short duration could contribute significantly to the control of this disease.

The performance of the vaccine in our trial on human volunteers was encouraging. With one exception (No. 11 in Table 1), the reactions in all subjects were mild or absent. Pain at the site of injection, which lasted for a 24-hour period, was noted in a few cases. The pain was most evident following the first injection and was markedly less following the second and third. The one exception with a generalized reaction following the second dose was a subject who had a pre-immunization titre of 1 : 1 024 by the bentonite flocculation test.

The results in the study have demonstrated clearly that the antibodies measured by the two tests—the bentonite flocculation test and the tissue culture inhibition test—are not the same. It was pointed out earlier that each of the four persons who failed to develop inhibitory antibodies developed measurable flocculating antibodies. Similarly, each of the 12 subjects who failed to develop flocculating antibodies had inhibiting antibodies in their post-immunization sera. The possible significance of the different antibodies measured has been referred to in the text.

Encouraging as these results are, it is obvious that the value of the vaccine can be proved only by a successful clinical field trial in man.

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