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Duration of the immune response in subjects inoculated with antimeningococcal A and C vaccines kept in storage at −20 °C and at 4 °C: influence of pre-vaccination titres on the vaccinal response*

By S. GRINSTEIN, T. M. KAHN, S. TISMINETSKY, MARTA DONADIO
Hospital de Niños 'Ricardo Gutiérrez', Servicio de Virología–Serología, Gallo 1330, 1425 *Buenos Aires, Argentina*
AND G. WEYLAND
Navy Hospital, Puerto Belgrano, Argentina

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SUMMARY

The antibody titres in 250 subjects, aged 5 to 22 years, who were vaccinated with a mannitol-lyophilized antimeningococcal A + C vaccine, stable only when stored at −20 °C, were followed for two years. As measured by indirect haemagglutination (IHA) and indirect immunofluorescence (IF) techniques, titres for both A and C *Neisseria meningitidis* antibodies remained high. Two years after vaccination titres of antibodies against type A showed fourfold increase over the initial titres in from 46 % to 100 % of groups of subjects and against type C in from 42 % to 80 %.

For 130 subjects vaccinated with a new lactose-lyophilized antimeningococcal A + C vaccine (presumed stable at 4 °C) antibody titres were measured up to 16 months after inoculation with this vaccine stored at −20 °C and also after storage for several periods at 4 °C. Antibody titres in all these subjects had fallen to their initial titres by 16 months.

The importance of evaluating the results on subjects showing low initial titres (⩽ 1/8 as measured by IHA) is discussed, as inclusion of high initial titres influences the extent of the response.

INTRODUCTION

The use of antimeningococcal A + C vaccines is recommended in high-risk populations (military barracks, institutions, etc.) and for the control of epidemics (Recommendation of the Public Health Service Advisory Committee on Immuniza-

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tion Practices, 1978), as the safety, effectiveness and protective capacity of these vaccines in adults and in children over two years old have already been accurately determined (Gold & Artenstein, 1971; Wahdan *et al.* 1973; Maekela *et al.* 1975).

This paper deals with two problems, neither yet fully clarified, relating to these vaccines. The first concerns the duration of the immunological response to vaccination; the second is concerned with the relatively unstable character of the preparations which necessitates storage at a temperature of −20 °C. To determine the duration of immunological response, a study was conducted on 250 subjects aged 5 to 22 years for two years after vaccination. The results of vaccination of 130 subjects aged 13 to 18 years with an experimental lactose-lyophilized preparation which, on account of its physicochemical properties, was considered to be presumptively stable at 4 °C, (Helting & Zwisler, 1977) are reported. This vaccine was administered after different periods of storage at 4 °C. Serum antibodies were determined up to one year after inoculation.

As evidence already exists of the relationship between serum antibodies and protection against meningococcal infection (Goldschneider, Gotschlich & Artenstein (1969), serological techniques were used to determine the presence of antibodies (Ab) against *N. meningitidis* types A and C.

The subjects selected for the trials were either members of semi-closed communities or recent naval recruits: all of whom from such populations are known to be particularly at risk of meningococcal infections. The subjects were drawn from the following sources:

(1) Stella Maris Navy Home (SM). Children of both sexes were assigned to two groups, SM1 ages 5 to 11 years; SM2 ages 12 to 16 years. These boarders from a low socio-economical level of the population sleep in 15-bed dormitories divided as to sex and attend primary school on the Home's premises or a secondary college outside. Many of these children spend the weekends away with their families.

(2) Admiral Brown Navy Lyceum (NL). The subjects selected were males who had joined in 1976 and 1977 aged 13 to 14 years. Subjects who joined in 1976 were selected five months after joining; those selected in 1977, three months after joining. All belonged to the medium- and high-income socio-economic groups with family homes either in Buenos Aires city or in surrounding localities. Each dormitory at the Lyceum holds 385 beds, each separated by one metre.

(3) Belgrano Harbour School of Nursing (BH). Students of both sexes were 17 to 22 years old when the study started. Male students board in the School; women students live at home thus constituting the female segment of an open population.

(4) The remaining subjects (CIPE) were male recruits, 18 years of age, who came from the provinces of Catamarca, La Rioja, Chubut and La Pampa to serve their conscription training period in the Argentine navy.

MATERIAL AND METHODS

Vaccines

Two vaccines were used: 'Vaccinum meningitidis cerebrospinalis A + C' batch no. 4 (Behringwerke, Federal Republic of Germany) which remains stable when stored at −20 °C and batch no. 8, presumed stable at 4 °C (Helting & Zwisler, 1977) by being lactose- instead of mannitol-lyophilized. The dose of each vaccine by subcutaneous injection was 50 μg of each polysaccharide.

Blood Samples

Blood samples were collected before and approximately one month, one year and two years after vaccination with batch no. 4. An additional sample was taken from SM subjects at 17 months. From subjects vaccinated with batch no. 8, samples were collected before and one month and one year after vaccination. All such were stored at −20 °C.

Antibody titres

Antibody titres (Ab) were determined by both indirect haemagglutination (IHA) and indirect immunofluorescence (IF) techniques. All samples belonging to any one subject were tested at the same time.

IHA

Tanned, formaldehyde-fixed type O, Rh-negative human red blood cells were used (Daniel, Weyand & Stavitzky, 1963), sensitized with the following purified polysaccharides used in the manufacture of the vaccines: Lot MP10 N.m.A. polysaccharide isolated from N.m.A 4 strain; and Lot MP7 N.m.C polysaccharide isolated from N.m.C 11 strain (Behringwerke, Federal Republic of Germany). Strains A4 and C11 were used for the IF determinations. The assays were performed using microdiluents and 0·025 ml burettes on disposable 'U' plates.

Each assay included positive rabbit antiserum controls, lot 60 anti-N.m.A and lot 34 N.m.C (Neisseria Repository, Naval Medical Research Unit no. 1, University of California, Berkeley, U.S.A.), as well as positive and negative human sera.

The titre was defined as the highest effective dilution producing agglutination of the red blood cells. (The number was the dilution tube number starting with tube no. 1 = one half dilution, successively undergoing one half dilution).

IF

The technique followed was that described by Artenstein *et al.* (1971) using strains A4 and C11 for determination of antibody titres. The strains were supplied by Behringwerke (Federal Republic of Germany) and their use was restricted to between two and five subcultures from the original lyophilized cultures. The working stock (two to three sub-cultures) was preserved in horse serum at −20 °C. Lot 587 goat total human anti-immunoglobulins (Immunochemia) was used at a dilution of 1/20. Titres were determined by epifluorescence through a Reichert

Table 1. *Subjects inoculated with batch no. 4, stable only at −20 °C*

(Subjects showing fourfold or greater increase in titre after inoculation)

		One month		Two years	
Antibody	Population	All subjects	Initial titre 1/8 or lower	All subjects	Initial titre 1/8 or lower
A	SM 1	64/31 (48)	1/1 (100)	50/1 (2)	1/1 (100)
	SM 2	48/28 (58)	1/1 (100)	13/1 (8)	1/1 (100)
	BH	54/14 (26)	2/2 (100)	22/1 (5)	2/1 (50)
	NL 76	84/69 (82)	79/69 (87)	52/21 (40)	48/21 (44)
C	SM 1	64/61 (95)	58/58 (100)	51/37 (73)	46/37 (80)
	SM 2	48/38 (79)	33/32 (97)	13/8 (62)	9/7 (78)
	BH	54/27 (50)	19/18 (95)	22/6 (27)	9/6 (67)
	NL 76	84/71 (85)	64/60 (94)	52/16 (31)	38/16 (42)

In all columns the numerator = total subjects in population group, denominator = number showing fourfold or greater rise in titre. Percentages in parentheses.

Fluorpan microscope equipped with 50 W HBA Lamps, 1·5 mm OGI and 1·0 mm GG9 blue ultraviolet absorption filters, 3 mm BG 12 excitator filter and 95X glycerine-immersion object lens. High titres, low titres and negative controls were used in each assay. The titre was defined as the highest dilution giving positive immunofluorescence. Student's *t*-test for paired samples was used for comparison of the means of IHA and IF titres in each population group. Student's *t* test for independent samples was used to compose the results obtained in males and females in two population groups.

Vaccination was considered effective when 80% or more of the subjects from each population group showed at least a fourfold increase in antibody titres. This criterion follows that advocated by the United States Food and Drugs Administration for the determination of bactericidal power, a technique which is, however, too complex and too expensive to use on a large scale.

RESULTS

Reactions

Adverse reactions to either vaccine were minimal, (mild erythema or erythema with oedema at the site of injection). Pyrexia in excess of 37·5 °C was noted in one subject only from the BH population.

Antibody

No significant difference was found at the 5% level between the results obtained by IHA and IF in 80 sera (antibody A) and 82 sera (antibody C), using Student's *t* test for paired samples (difference of means in standard deviation units = 1, approximately 95% confidence limits). Correlation coefficients of $n = 0{\cdot}9401$ for antibody A and 0·8334 for antibody C were attained.

Using Student's *t* test for unpaired samples, no significant differences at the

Table 2. *Subjects inoculated with batch no. 8, presumed stable at + 4 °C after different periods of storage*

(Subjects showing fourfold or greater increases in titre after inoculation)

		One month		Sixteen months	
Antibody	Population	All subjects	Initial titre 1/8 or lower	All subjects	Initial titre 1/8 or lower
A	CIPE 0*	27/11 (41)	9/6 (67)	ND	ND
	CIPE 7	25/18 (72)	16/15 (94)	ND	ND
	CIPE 22	16/2 (13)	6/1 (17)	ND	ND
	CIPE 37	24/5 (21)	9/5 (56)	ND	ND
	NL 77 120	38/23 (61)	14/13 (93)	32/1 (3)	14/1 (7)
C	CIPE 0	27/12 (44)	14/12 (86)	ND	ND
	CIPE 7	25/16 (64)	21/16 (76)	ND	ND
	CIPE 22	16/9 (56)	9/7 (78)	ND	ND
	CIPE 37	24/17 (71)	12/11 (92)	ND	ND
	NL 77 120	38/24 (63)	30/23 (77)	32/0	28/0

* Days vaccine stored at 4°C before use. 0 indicates storage at − 20 °C; ND = not done. See footnotes to Table 1.

5% level were observed in response by sex (SM and BH populations) at either one month or two years after inoculation.

Very high initial titres of A antibodies were found in subjects belonging to the SM populations (population mean 5·80; standard deviation 1·23). BH subjects showed high titres of C antibodies. NL subjects had low titres for both A and C antibodies. (For discussion of these titres and those found in the CIPE population, see Kahn & Grinstein, 1978.)

The data in Table 1 shows separately all subjects with low initial titres, defined as titre 3 or lower ($\leqslant$ 1/8) for the reason that most subjects who showed a higher initial titre either failed to react to the vaccine or showed an increase of only one dilution (Kahn *et al.* 1978). These results agree with those of Artenstein *et al.* (1971) who used the same IHA technique. No decrease in initial titre was found one month after vaccination in subjects inoculated with either vaccine, irrespective of the initial titre, whether high or low.

It is apparent from Table 1 that the results obtained with vaccine no. 4 one month after inoculation satisfied the criterion of effectiveness described above for both anti-N.m. A and C antibodies. The results obtained with vaccine no. 8, presumed stable at 4 °C, were irregular (Table 2), for only in some instances did 80% of subjects show a fourfold increase in titre. The period of storage of the vaccine at 4 °C before inoculation did not appear to influence the results.

The most outstanding difference between the batches of vaccine appeared to be that, while for vaccine batch no. 8 the maximum increase in titre among subjects showing an increase greater than fourfold was eightfold, subjects inoculated with vaccine batch no. 4 exhibited on average a rise of 16 times. One subject from population SM even showed a rise in titre to 1/2048. With both batches of vaccine the greatest rises in titres were seen with the lowest initial titres (Kahn *et al.* 1978).

Scatter chart 1. *SM population, antibody A*

(Titre* difference two years after inoculation from initial titre)

Initial titre	−4	−3	−2	−1	0	1	2	3	4	Total
1 or lower									1	1
2										
3							1			1
4						3	2			5
5				2	10	2				14
6		2	7	9	8					26
7		4	3	3	2					12
8	1	3								4
Total	1	9	10	14	20	5	3	—	1	63

* Titre expressed as tube number: initial dilutions $\frac{1}{2}$, successive doubling dilutions.

Scatter chart 2. *NL 76 population, antibody C*

(Titre* difference two years after inoculation from initial titre)

Initial titre	−4	−3	−2	−1	0	1	2	3	Total
1 or lower						5	7	4	16
2					2	7	4		13
3					2	6	1		9
4				2	4				6
5		1	1	1	3				6
6	2								2
Total	2	1	1	3	11	18	12	4	52

* Titre expressed as tube number: initial dilutions $\frac{1}{2}$, successive doubling dilutions.

Titres of sera collected 16 months after vaccination with batch no. 8 from NL 77 subjects only (CIPE subjects returned to civilian life after about 1 year of military service) show a reversion to initial titres (Table 2).

In contrast, titres of sera collected 2 years after vaccination with batch no. 4 shows that for A antibodies 44% of subjects from population NL 76 and for C antibodies 42% to 80% of subjects in different population groups still showed fourfold or greater rises over initial titres of $\leqslant 1/8$ (Table 1; scatter charts 1, 2). Considering the total number of the three groups vaccinated with batch no. 4, 24 out of 52 subjects (46%), and 66 out of 102 subjects (65%) two years after inocula-

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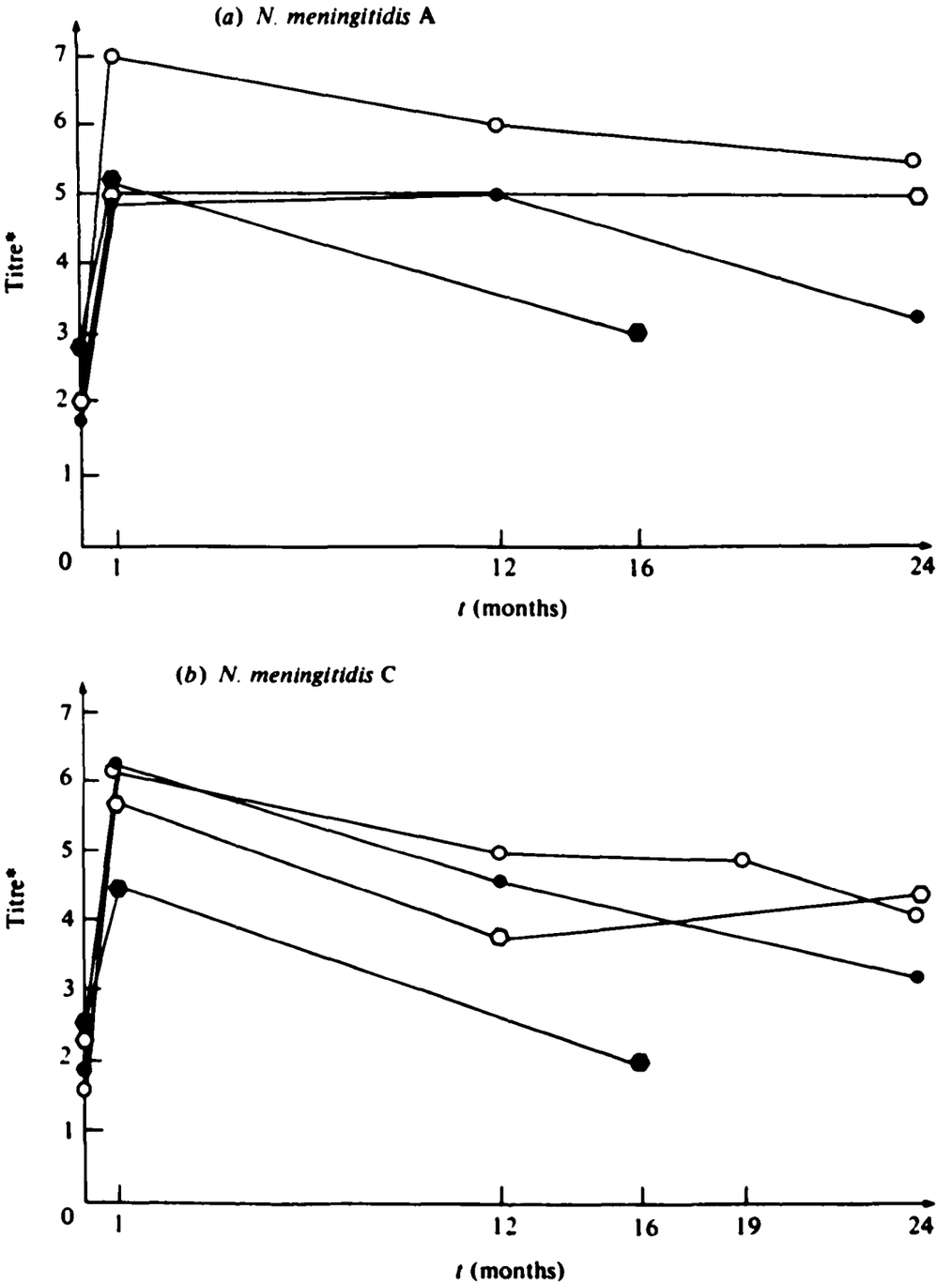

Fig. 1. Average level of anti-*N. meningitidis* A and C antibodies in subjects whose initial titre was 3 or lower before ($t = 0$) and at different periods after vaccination. SM (○), NL 76 (○) and BH (●): inoculated with vaccine batch no. 4, stable only at −20 °C; NL 77 (●): inoculated with vaccine batch no. 8, presumed stable at 4 °C, stored for four months at 4 °C. *Figures refer to tube in doubling dilution series.

tion still showed a fourfold or greater increase in titres for antibodies against types A and C respectively.

Furthermore, considering total subjects with low initial titres, vaccinated with batch no. 4, 86% and 94% exhibit one or more titre increases for antibodies against types A and C two years after inoculation (data for one or more increases not shown in tables).

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Fig. 1 shows the steady decline in antibody titres during the two years follow-up. The difference between population NL 77, vaccinated with batch no. 8, in which the titres had fallen to their initial values 16 months after inoculation, and the other three population groups is indeed significant, as the latter maintain, at the very least (population NL 76) an average of 1·4 (type A antibody) and 1·3 (type C antibody) dilution titres higher than the initial titres two years after inoculation.

DISCUSSION

The results obtained with vaccine batch no. 8, presumed stable at 4 °C, when inoculated after storage at this temperature for different periods were consistently poorer than those obtained with vaccine batch no. 4, stable only when stored at − 20 °C, at both one month and 16 months after inoculation. At 16 months, titres had fallen to their pre-vaccination levels. Storage of vaccine batch no. 8 at − 20 °C did not alter this finding. Vaccine batch no. 8, which was lactose- instead of mannitol-lyophilized, had, however, been purified more extensively than usual which caused its pyrogen content to be lower than that of vaccine batch no. 4. Since group A polysaccharides maintain their high molecular weight as well as their ability to react with antibody *in vitro* even when stored under more disadvantageous temperature conditions than those applied in this study, it would appear that the results obtained might be due to the purification procedures used (Tiesjma, Beuvery & Te Par, 1977). A further fact pointing in this direction is that the results were not affected by the duration of storage at 4 °C. It should be stressed, however, that neither the high molecular weight, nor an antigen content comparable to that of the traditional vaccine when examined by an *in vitro* enzyme-linked immunosorbent assay is by itself guarantee for assuring immunization in man. It would seem necessary, then, to conduct tests on human subjects, like those carried out in this study, when purification of the vaccine undergoes revision.

By using IHA and IF techniques an outstanding difference between vaccine batches no. 4 and no. 8 has been shown. Brandt, Artenstein & Smith (1973) using these techniques to assess the loss of potency of several lots of antimeningococcal A vaccine after long-term storage, failed to detect significant variations but did detect them by use of the radioactive antigen-binding assay method (RABA). Our detection of the differences found may be ascribed to one or other of the following considerations:

(1) The use of red blood cells stabilized before sensitizing rendered our IHA test slightly more sensitive than that in which fresh red blood cells were used (unpublished observations). In addition, stabilized, sensitized red blood cells could be stored for more than six months without noticeable loss in titre, thus ensuring comparability of results.

(2) The differences between the vaccine batches studied were so great that the IHA technique, although of undoubtedly lower sensitivity than that of the RABA method, was able to detect them.

(3) In analysing their findings Brandt *et al.* (1973) failed to distinguish between

subjects with high or low initial titres. In our analysis, this distinction is made resulting in easier comparisons.

There are few papers which describe long-term follow-up of subjects after inoculation with antimeningococcal vaccines. Brandt & Artenstein (1975) present the longest follow-up known to us. This study, covering a 5-year period is, however, limited in the sense that it refers to the persistence of only antibody A in 23 young adult volunteers. In the present study, we have dealt with 250 subjects, children aged five and over and young adults, all inoculated with a combined A & C vaccine (batch no. 4, −20 °C). Our results agree with those reported by Brandt *et al.* (1973) in that, for the duration of the study, we observed persistence of antibodies A and C at much higher levels than initially; in the most favourable case, SM1, 80% of children show a fourfold or greater rise in titre over initial titres two years after vaccination (antibody C, Table 1).

We do not know whether these antibodies confer protection against infection since protection can be assessed only by testing the bactericidal ability of the blood or by evaluating in time the occurrence of infection in vaccinated and control subjects. This latter procedure could not be adopted, as very few infections were reported in the general population during the period of the study. In addition, no infections were reported either in vaccinated subjects or in a field survey covering about 25000 men enlisted in the Argentine Navy (Grinstein & Schwartz, 1977).

In other studies it has been reported that up to 15% of subjects who possess antibodies detected by IHA techniques lack bactericidal antibodies and, further, that subjects who possess more than 50 ng of binding antigen as measured by RABA also lack bactericidal antibodies, this latter despite the good correlation between both techniques and the RABA method (Brandt *et al.*, 1973; Brandt & Artenstein, 1975). Thus, assuming that even 15% of our study subjects who were shown to possess antibodies by IHA and IF techniques lacked bactericidal antibodies, our findings indicate not only good persistence but, most probably, a protective ability for a substantial percentage of subjects vaccinated with batch no. 4. This conclusion agreed fully with results obtained in Egypt after anti-N.m.A vaccination where protective cover lasting for one to three years (dependent on the batch of vaccine used) was achieved (Whadan *et al.* 1973, 1977).

The IHA and IF correlation coefficients for anti-N.m.A and C antibodies are high ($n = 0{\cdot}9401$ and $n = 0{\cdot}8334$ respectively), showing that the high initial titres detected by IHA are not the result of technical error. It should be remembered that these techniques are not two different methods for evaluating the same antibody. The IHA technique detects only antibodies against the specific polysaccharide groups of N.m.A and C, while the IF technique detects, in addition to these antigens, other surface antigens possessed by *N. meningitidis*. Thus, in two subjects of the SM population (C antibody) IF showed a large increase in titre of the sample taken two years after vaccination, while the IHA titres showed a continuous decrease at a rate of about one dilution by half per year. We surmise that both these subjects had suffered infection with some microorganism which, although lacking the specific group polysaccharides of N.m. group C, did possess

some surface antigen or antigens shared by the N.m.C11 used in our IF determinations.

We were unable to obtain retropharyngeal control swabs. We consider, however, that meningococcal A infection was unlikely to have occurred as no infections had been reported during the last three years in the community (Kahn & Grinstein, 1978; authors' unpublished findings).

It is possible, of course, that some subjects could have been carrying C meningococci during the study but the antibody levels after inoculation do not support this suggestion since, with the exception of the two subjects discussed above, the antibody levels of all subjects who reacted to the vaccine either fell gradually or remained unchanged. In this connection, however, Brandt & Artenstein (1975) have reported that subjects in a vaccination study became meningococcal carriers during the study without showing any increase in their antibody levels.

Particular attention has been paid to the initial antibody titre. Only subjects with low initial titres were useful for evaluating these vaccines, because they are able to fulfil the criterion of effectiveness defined as a fourfold or greater rise in initial titres. Given a high initial titre, the response to inoculation is generally poor. Assuming that the rate of elimination of antibody is equal to that seen in subjects showing a more efficient initial response, the initial titre will be reattained sooner. Significantly, the titres in subjects with high initial titres had fallen to up to one sixteenth of the initial titre two years after vaccination.

The high initial titres found, particularly those against A antigen, are considered to have been produced not in response to infection with *N. meningitidis* group A, but in response to subclinical infection with other bacteria of which some antigens show cross-reactions with the polysaccharides typical of group A (Robbins *et al.* 1972; Myerowitz, Gordon & Robbins, 1973; Vann, Liu & Robbins, 1976). We assume that high initial titres of group C antibody can be attributed in similar fashion although in this case subclinical infection with *N. meningitidis* cannot be excluded.

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