Br. J. exp. Path. (1978) **59**, 544

FURTHER STUDIES ON THE IMMUNOLOGY OF THE INFANT RAT EXPERIMENTAL MODEL OF *HAEMOPHILUS INFLUENZAE* TYPE B MENINGITIS

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Received for publication May 22, 1978

Summary.—An age-related acquisition of anticapsular (AC) antibodies to *Haemophilus influenzae* b (HIb) did not occur in rats; this result corrects a previous spurious observation from this laboratory. Purified HIb polysaccharide was found to be totally non-immunogenic over a wide range of doses in infant and adult rats, with or without booster immunizations. Killed HIb was also totally non-immunogenic. Only about one-quarter of survivors of infantile HIb disease made an AC antibody response. In an attempt to observe "immune paralysis", survivors of infantile HIb disease were immunized with live HIb at age 8 weeks. This immunization resulted in significantly fewer AC antibody responders in the survivors than in the controls. However, immunization of survivors with killed HIb produced significantly greater responders in survivors than in controls. In order to determine whether passive protection from HIb disease could be achieved by non-capsular antibodies, survivors' offspring were studied and found to be protected from infantile HIb disease due to bactericidal (BA) antibodies that were probably not directed at the capsular polysaccharide. Using the BA assay, differences were observed between two HIb strains, presumably due to differences in non-capsular antigens.

The infant rat experimental model of HIb disease appears to have only limited value in the study of AC antibody responses since infant rats differ from infant humans in several respects. The model may be more useful in studying the immunology of non-capsular HIb antigens including their role in protective immunity and possible differences in these antigens among strains of HIb.

IMMUNITY to *Haemophilus influenzae* type b (HIb) was originally shown by Fothergill and Wright (1933) to be age-related and to be due to bactericidal (BA) antibodies. Subsequently, Schneerson *et al.* (1971) and others demonstrated that many of these BA antibodies were directed at the type b capsular polysaccharide, although the possibility that other antigens are important in protective immunity has not been fully evaluated. Recently, attempts to induce immunity to HIb in infants have been carried out with purified capsular polysaccharide vaccine. However, Parke *et al.* (1976) have shown that this vaccine is not very immunogenic in human infants. Also, the observation by Norden and Michaels (1973) that survivors of HIb meningitis do not respond with anticapsular (AC) antibodies as well as controls to subsequent immunization with this vaccine has raised the possibility of "immune paralysis" which might be induced by immunization with the vaccine in infancy.

For all these reasons, several questions concerning immunity to *H. influenzae* Type b (HIb) have emerged which require study both in humans and experimental animals. (1) Does age-related immunity to HIb, similar to that of humans, occur in rats, a species for which a reproducible experimental model of HIb disease has recently been developed by Moxon *et al.*

(1974)? (2) Can "immune paralysis" to the capsular polysaccharide of HIb, a phenomenon whereby exposure of the young individual to large amounts of polysaccharide antigen in early life results in specific suppression of immunity to that antigen in later life, be experimentally reproduced in rats following infantile HIb infection? (3) Is the purified form of the HIb capsular polysaccharide immunogenic in any form in infant humans or rats? (4) What is the role of antibodies directed against antigens other than the HIb capsular polysaccharide in natural and post-infection immunity to HIb disease, *i.e.* are such antibodies protective against subsequent infection by HIb? (5) Is the AC antibody response of infant rats to HIb disease analogous to that of humans?

The present investigation was designed to extend the previous observations of Myerowitz and Norden (1977*a*) and to investigate these questions further using the infant rat experimental model of HIb disease. The issue of age-related natural immunity to HIb in rats was re-investigated using a single lot of radioactive antigen with high specific activity. The immunogenicity of purified capsular polysaccharide antigen in rats was systematically studied using various doses in both infant and adult rats. The immunogenicity of this antigen on a large particle (whole killed HIb) was also studied. Survivors of infantile HIb disease were studied to determine both the incidence of AC antibody responses and whether subsequent immunization by a variety of modes at an early age (8 weeks) could demonstrate that "immune paralysis" had occurred in survivors as compared to controls. Finally, the offspring of survivors were studied to determine if they were indeed protected from experimental HIb disease and whether this protection was due to AC antibodies or non-capsular BA antibodies.

MATERIALS AND METHODS

Animals.—Outbred, pathogen-free, albino Sprague–Dawley rats were purchased from Hilltop Lab Animals, Scottsdale, Pa. and housed in a single room at the Children's Hospital of Pittsburgh.

Inoculation and culture of animals.—The techniques used for i.p. and intranasal (i.n.) bacterial inoculation of rats and those used for quantitative culture of blood have been previously described by Myerowitz and Norden (1977*b*).

Bacteriological methods.—The technique of storage and culture of HIb strains has been previously described by Myerowitz and Norden (1977*b*). For the present experiments, HIb strain Pekala had been passaged 5 times through infant rats by i.p. inoculation and recovery from blood within 24 h. For BA assay, both this strain and Strain Decker were used. Strain Decker was originally isolated from the spinal fluid of a child with meningitis and has been used for several years as a stock strain for BA assays in our laboratory. Killed HIb for immunization were prepared by resuspension of an overnight Leventhal broth culture in 2% phosphate-buffered formalin. After incubation for 18 h at 4°, the bacteria were centrifuged and resuspended 3 times in phosphate-buffered saline (PBS, 0·05M, pH 7·1).

Immunological methods.—Serum AC antibody concentrations were determined by the radioactive antigen-binding assay as described by Norden and Michaels (1973). For all experiments described in this report, a single lot of high mol. wt ^{125}I-labelled antigen was used. This lot of antigen gave a highly reproducible binding curve and unlabelled polysaccharide antigen completely blocked the binding of labelled antigen by AC antibody. Using our standard serum, the sensitivity of the assay with this antigen lot was 70 ng of antibody protein per ml of serum. Antibody determinations were done in duplicate and all assays were performed within a period of 2 weeks during which the antigen maintained a high specific activity. BA antibodies were determined as described by Norden *et al.* (1972). Results are expressed as positive (>70% killing), weakly positive (45–70% killing), or negative (<45% killing).

Experimental design.—In order to determine whether an age-related acquisition of "naturally acquired" HIb AC antibodies occurred in rats, a single litter of 13 rats was raised in our animal quarters and bled by cardiac puncture at the ages of 2, 4, 6, 8, 10 and 12 weeks. Since extensive previous investigations had shown that pharyngeal or enteric colonization with HIb or cross-reacting bacteria almost never occurred in our colony, we did not monitor these rats bacteriologically. All sera from offspring were tested for AC and BA antibodies except the sample at 2 weeks of age, which was tested only for AC antibody, owing to the small volume of the sample.

The immunogenicity of purified polysacchar-

TABLE I.—*Experimental Protocol to Study the Immune Response of Survivors of Infantile* Haemophilus influenzae *Type b Bacteraemia*

Group*	1	2	3	4	5
Age					
5 days	10^2 HIb i.p. (experimental set) or saline i.p. (control set)				
7 days			Blood culture		
7 weeks			Sera obtained		
8 weeks	10^4 live HIb i.p.	10^9 killed HIb i.p.	0·01 μg† s.c.	0·1 μg† s.c.	1·0 μg† s.c.
10 weeks			Sera obtained		

* Each group within experimental and control sets consisted of 10 rats.
† Dosage of purified Type b capsular polysaccharide antigen.

ide vaccine was studied as follows; individual litters (ranging in size from 8 to 14 rats) were immunized with either 0·001 μg, 0·01 μg, or 0·1 μg purified polysaccharide s.c. at the age of 5 days. Litters were bled when aged 3 weeks. A saline inoculated litter was used as a control. Booster effect was studied by re-inoculating these litters at the age of 3 months with 0·01, 0·1, and 1·0 μg of polysaccharide s.c. respectively and bleeding 2 weeks after vaccination. Three other litters not previously vaccinated were similarly immunized at the age of 3 months. The immunogenicity of polysaccharide antigen on a large particle was studied as follows; one litter was immunized at 5 days of age with 10^7 killed HIb i.p., bled at the age of 3 weeks, re-immunized at 3 months with 0·1 μg of polysaccharide s.c., and bled 2 weeks later. A separate litter was immunized with 10^9 killed HIb only at the age of 3 months. All sera were tested for AC antibodies. Only the sera from rats immunized with killed HIb were tested for BA antibodies.

The protocol used in the first experiment to study the immune response of survivors of infantile HIb disease is shown in Table I. For this experiment 2 sets of rats, an experimental and control set, each with 5 litters of rats, were obtained simultaneously, pooled, and randomly alotted to the various groups. The experimental set received 10^2 HIb i.p. at 5 days of age; the control set received saline. All 50 experimental rats had $>10^4$ cfu/ml of HIb in their bloodstream at 48 h; all but 2 survived the episode. None of the saline controls were bacteraemic. The 5 groups within each set differed only in the nature of the immunogen used at the age of 8 weeks. The second experiment was identical to the first except that only Groups 1 and 2 (live and killed HIb) were studied.

In order to study the offspring of survivors of infantile HIb disease, 9 female rats who had survived i.p.-induced HIb disease at the age of 5 days, were mated at about 10 weeks with one of two adult males derived from a separate litter. Litters were challenged when aged 5 days by either i.n. or i.p. inoculation and mothers and pups were bled for culture and serum 48 h after challenge. The sera from 3 pups in each litter were pooled to provide a large enough volume for AC and BA antibody determinations. Maternal and pup sera from the control litters were also tested for AC and BA antibodies.

RESULTS

Age-related development of AC and BA antibodies

All sera from the 13 rats bled twice weekly from the ages of 2 to 12 weeks had no detectable (<70 ng/ml) AC antibodies. The results of BA antibody assays were more variable. Five rats had BA activity while 8 had none at the age of 4 weeks. Five remained negative throughout the period of study and 3 developed BA antibodies by the age of 8 weeks. The results of these BA assays were similar using HIb Strain Pekala or Strain Decker, *i.e.* BA activity to Strain Decker was present in 33/62 (53%) sera as compared to 28/62 (45%) for Strain Pekala. Of 33 sera with BA activity to Strain Decker, 28 also had activity to Strain Pekala. Of 28 sera with BA activity to Strain Pekala, 25 also had activity to Strain Decker.

Immunogenicity of the HIb capsular polysaccharide antigen

All sera from infant and adult rats immunized with the various doses of purified polysaccharide, with or without booster immunization (see *Experimental Design*), had no detectable AC antibodies. Infant rats immunized with killed HIb had no detectable AC antibodies, but one of 9

3-month-old rats immunized with killed HIb had a barely detectable concentration of AC antibodies (80 ng/ml). The infant and adult rats which were inoculated with killed HIb had no detectable BA antibodies to either HIb Strain Pekala or Decker at 3 months of age including the rats which also received a polysaccharide booster immunization.

AC and BA antibody responses of survivors of infantile HIb disease

In the first experiment, 14 (29%) of 48 rats which survived HIb bacteraemia at the age of 5 days had detectable levels of AC antibodies at 7 weeks of age and are, therefore, assumed to have made an AC antibody response. Nine of 14 responding rats had low serum concentrations ranging from 100 to 370 ng/ml. The 5 other responding rats had levels greater than 1000 ng/ml (range=1050–3500 ng/ml). None of the 49 control rats had detectable AC antibodies before immunization. None of 10 survivors which were subsequently immunized with live HIb made an AC antibody response, compared to 7 of 9 similarly immunized controls which did make an AC antibody response (Table II). This difference is statistically significant ($P=0{\cdot}01$ using Fisher's exact test). Immunization with killed HIb or the various doses of purified polysaccharide antigen resulted in a low frequency of AC antibody responders and no differences were observed between survivors and controls.

TABLE II.—*Survivors of Infantile HIb Bacteraemia: Frequency of AC Antibody Responders After Various Forms of Immunization*

Immunogen	Survivors	Controls
Experiment No. 1		
10^4 live HIb i.p.	0/10*	7/9
10^9 killed HIb i.p.	3/10	1/10
0·01 μg† s.c.	0/9	0/10
0·10 μg s.c.	1/9	1/10
1·0 μg s.c.	2/10	1/10
Experiment No. 2		
10^4 live HIb i.p.	4/10	3/9
10^9 killed HIb i.p.	3/10	0/10

* Number of responders/total tested. A responder is defined as a greater than two-fold increase in AC antibody concentration or the appearance of a detectable concentration of antibodies in a rat with previously undetectable levels.

† Dose of purified capsular polysaccharide.

In an attempt to confirm that survivors of infantile HIb disease fail to respond with AC antibodies to live HIb at 8 weeks of age, *i.e.* that these rats exhibit "immune paralysis" to this form of immunization, a second group of experimental rats and control rats was tested using live and killed HIb as immunogens. Five (25%) of 20 survivors had a detectable AC antibody concentration at 7 weeks of age, all of which were at a low level (70–370 ng/ml). However, the results of this experiment (Table II) were different from the first in that the frequency of responders to immunization with live HIb among survivors (4/10) was almost identical to that of controls (3/9).

When the data for the 2 experiments were pooled, 2 interesting facts emerged. (1) Of the 68 5-day-old rats which survived infection with live HIb, 19 (28%) developed AC antibodies. However, of 18 7-week-old rats which survived infection, 10 (55%) developed AC antibodies ($\chi^2=4{\cdot}86$, $P<0{\cdot}05$). These data suggest that the AC antibody response of rats to initial infection with live HIb is age-related. (2) The frequency of AC antibody responders among survivors of infantile HIb disease when re-challenged with live HIb (4/20) was significantly decreased compared to controls (10/18, $P=0{\cdot}02$ using Fisher's exact test). However, the frequency of AC antibody responders among survivors challenged with killed HIb (6/20) was significantly increased compared to controls (1/20, $P=0{\cdot}04$ using Fisher's exact test).

BA antibodies to HIb Strain Pekala (the infecting strain) were present in all 48 survivors' sera in the first experiment at 8 weeks of age. Thirty-eight sera were positive and 10 were weakly positive. However, half of the survivors' sera had

TABLE III.—*Survivors of Infantile* Haemophilus influenzae *Type b Bacteraemia: Resistance of Offspring to Experimental HIb Bacteraemia at the Age of 5 Days and Correlation with Anticapsular and Bactericidal Antibodies*

Litter No.	Inoculum	Route	No. Bacteraemic/ No. Tested	Anticapsular antibodies*		Bactericidal antibodies (Pekala)						Bactericidal antibodies (Decker)					
				Maternal	Offspring	Maternal			Offspring			Maternal			Offspring		
						+	±	−	+	±	−	+	±	−	+	±	−
Experimental 1–7	10^8	i.n.	1/59	1/7	0/7	7	0	0	2	2	3	3	2	0	0	1	6
Control 8–10	10^8	i.n.	3/16	0/3	0/3	0	1	2	0	0	3	0	0	3	0	0	3
Experimental 11–12	10^2	i.p.	0/20	0/2	0/2	1	1	0	0	2	0	1	0	1	0	0	2
Control 13	10^2	i.p.	10/10	0/1	0/1	1	0	0	0	0	1	0	0	1	0	0	1

* Number of sera with detectable AC antibodies/total tested.

no BA activity to Strain Decker and only 11 sera were positive. This difference in BA activity of sera to Strain Pekala and Strain Decker was statistically significant ($\chi^2=39{\cdot}3$, $P<0{\cdot}0001$).

Susceptibility of survivor's offspring to experimental HIb disease

A total of 9 litters born to female survivors of infantile HIb disease were challenged with HIb at the age of 5 days (Table III). Of 59 rats from among 7 litters, one was bacteraemic at 48 h after i.n. inoculation of HIb and that rat had the lowest detectable concentration of bacteria (10^2 cfu/ml). Three of 16 control rats were bacteraemic with high-grade ($>10^4$ cfu/ml) concentrations of bacteria. The difference between experimental and control groups was significant (χ^2 with Yates correction$=4{\cdot}27$, $P<0{\cdot}05$). None of the 20 rats from among 2 litters were bacteraemic after i.p. inoculation compared with 10/10 control rats ($\chi^2=25{\cdot}7$, $P<0{\cdot}0001$). The protection from HIb disease among these offspring did not correlate with maternal or endogenous AC antibodies, which were present in only one of the 9 mothers, none of the offspring and none of the controls. The protection did correlate with BA antibodies to HIb Strain Pekala, which were positive in 8 of 9 mothers and positive or weakly positive in 6 of the 9 offspring sera, compared with one of 4 control mothers and none of the 4 control offspring ($P=0{\cdot}05$ using Fisher's exact test for mothers and offspring considered separately). Again, a striking increase in BA activity was observed in these sera to HIb Strain Pekala (the original infecting strain) compared with HIb Strain Decker. Of the 9 maternal sera, 8 were positive to Strain Pekala and only 4 to Strain Decker. Of sera from the 9 offspring, 6 were positive or weakly positive to Strain Pekala, whereas only one was weakly positive to Strain Decker and the other 8 sera were negative.

DISCUSSION

The results of this study have shown that the age-related acquisition of AC antibodies, which occurs in humans, does not occur in rats. While this conclusion is based primarily on study of a single litter of 13 rats, it should also be pointed out that, in the immune paralysis experiments, 68 control rats' sera were tested for AC antibodies at the age of 7 weeks and none were found. The previous results of Myerowitz and Norden (1977*a*), which indicated that such age-related acquisition did occur in rats, were due to the use of various lots of radioactive antigen for testing of sera at different ages. Subsequently it was learned that non-specific binding, as measured by the ability of unlabelled antigen to block binding, occurred with some of these lots. This non-specific binding presumably accounted for the initial spurious result. The

use of a single lot of radioactive antigen with high specific activity and no non-specific binding encourages us to believe that the present results are definitive.

Purified HIb polysaccharide antigen, which has been shown by Schneerson *et al.* (1971) to be highly immunogenic in human adults and by Parke *et al.* (1976) to be poorly immunogenic in human infants, appears to be completely non-immunogenic in infant or adult rats. Furthermore, killed whole HIb, which represents capsular antigen attached to a large particle, was also non-immunogenic in infant or adult rats. Only active infection with live HIb is immunogenic at all with respect to the stimulation of AC antibodies and, in infant rats, only one-third of survivors developed an AC antibody response.

Immunization of survivors of infantile HIb disease at 8 weeks of age with live bacteria resulted in a significantly decreased incidence of AC antibody responses when compared to controls. This apparent "immune paralysis" differed from our previous experience with 17-week-old rats who were able to mount an AC antibody response with a similar high frequency as in controls. However, immunization of survivors with killed HIb resulted in a significantly increased incidence of AC antibody responses when compared to controls. These conflicting results indicate that further studies in survivors of different ages are required to define whether immune paralysis can be reproducibly demonstrated in rats using the HIb capsular polysaccharide antigen.

The studies of survivors' offspring confirm the results of Granoff and Rockwell (1978) that such offspring are protected from HIb disease and that this protection correlates with the presence of BA antibodies. Since the maternal and offspring sera had no detectable AC antibodies, it is assumed that the BA antibodies are directed against non-capsular antigens. This assumption is strengthened by the fact that the level of AC antibodies needed to give a positive result in the BA assay (600 ng/ml) is well above the limit of detection of our AC antibody assay. Granoff and Rockwell (1978) have also shown that absorption of survivors' sera with purified Type b antigen fails to reduce BA activity. These data strongly support the idea that non-capsular antibodies may be protective against HIb disease, which was also supported by our previous passive protection experiments and by other investigations of BA antibodies such as that of Mpairwe (1971).

During our study of BA activity of sera from survivors and their offspring, it became apparent that BA activity directed against the infecting strain (Pekala) was significantly greater than the activity directed against a laboratory stock strain (Decker). Such strain differences are presumably due to differences in non-capsular antigens and these differences have been previously noted by Norden (1972). Further study is required to determine if such differences are significant with respect to protection against experimental and natural disease. Experiments are under way to determine if survivors' offspring previously infected with Strain Pekala will also be protected against Strain Decker, and *vice versa*.

In conclusion, the infant rat experimental model of HIb disease appears to have only limited usefulness as a model for studying immunity to the Type b capsular antigen. Immunity to this antigen in rats differs from humans in several respects; (1) Rats do not develop an age-related increase in AC antibodies, perhaps because of the lack of enteric colonization with cross-reacting bacteria. (2) Purified antigen is totally non-immunogenic even in adult rats. (3) Immune suppression in survivors of infantile HIb disease may not occur in rats. (4) The frequency of AC antibody responses following infantile infection is much lower in infant rats than in infant humans. This model may have considerably more usefulness in studying the role of non-capsular antigens in immunity to HIb. Using passive protection acquired either artificially or through immunization of mothers, the nature and

specificity of these antigens may be defined. Also, possible strain differences in non-capsular antigens among HIb should also be evaluated.

We wish to thank Drs John B. Robbins and Rachel Schneerson of the Bureau of Biologics for supplying radiolabelled capsular antigen, Ms Cheryl Myerowitz for secretarial assistance, and Mr Thomas Demchak and Ms Heidi Wentzel for technical assistance.

This work was supported by Grant S5 from the Health Research and Services Foundation of Pittsburgh and by Grant No. R01 AI-11932-04 from the National Institutes of Health.

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