Accelerated and Prolongated Multiplication of Antibody-Forming Spleen Cells by *Bordetella pertussis* in Mice Immunized with Sheep Red Blood Cells

It has been shown that addition of *Bordetella pertussis* to toxoid from *Corynebacterium diphtheriae* produced an enhancement of antitoxin formation in guinea-pigs[1-3] and likewise in children[4]. It is also well known that pertussis vaccine can be employed as an immunological adjuvant for increased development of experimental 'allergic' encephalomyelitis[5-11]. Above all *B. pertussis* has been used for the induction of susceptibility to fatal systemic anaphylaxis in mice and rats, which in general is much more difficult to produce with these species than with other species of experimental animals used. After a single injection of antigen, prompt susceptibility to anaphylaxis is only induced in several inbred strains of mice when pertussis cells are given together with the sensitizing protein antigen[12-15] or when the antigen is applied as a water-in-oil-emulsion prepared with Freund's adjuvant[14,15]. Previous investigations have shown that the treatment of mice with pertussis vaccine increases the production of agglutinins against chicken red blood cells[16] and of hemagglutinating antibodies to protein antigens[14,15]. Thus it was of interest to find out whether such action is connected with an increase of cellular activity resulting in the augmentation of circulating antibodies by a constant number of immunologically competent cells, or just with a multiplication of antibody producing spleen cells developing concomitantly with the marked elevation of the individual spleen weights found in pertussis-treated mice[17,18].

For the quantitative determination of plaque-forming spleen cells, the agar technique as described by JERNE et al.[19,20] was employed. Suspensions of single spleen cells were prepared as described[20] using Eagle's medium. The suspensions were filtered through a sterile, fine-mesh gauze. Cell counts were performed microscopically in a Bürker-chamber using a 1:200 dilution of the test spleen cell suspension. The actual dilution of the spleen cell suspensions taken depended on the anticipated proportion of plaque-forming cells. For pouring agar plates, cell suspensions were used containing $5.5 \cdot 10^6$ to $4.8 \cdot 10^7$ nucleated cells per ml. In brief, 0.1 ml of the test spleen cell suspension was added to 2.0 ml of melted 0.7% Oxoid agar (No. 3) containing $4 \cdot 10^8$ sheep red blood cells (SRBC) and 1.0 mg diethylaminoethyl-dextran. This mixture was carefully poured over the basal 3 mm thick layer of agar, previously prepared in petri dishes (80 mm in diameter). After the upper cell agar layer had solidified, the plates were incubated at 37 °C for 1 h. Subsequently, 1.5 ml of undiluted guinea-pig complement was spread over the surface of the plates and incubated for 30 min at 37°C. Following this incubation, plates were allowed to stay for 60 min at room temperature and then stored at 4°C in a refrigerator for 12-15 h. They were carefully examined for plaques in the layer using a plate-microscope. For the experiments male albino mice of the inbred strain NMRI/Han. were used.

In a preliminary first investigation 80 mice were divided into 3 groups. The 25 mice of group I received only a single i.v. injection of $4 \cdot 10^6$ SRBC, while the 25 mice of group II simultaneously with the i.v. injected SRBC, received an i.p. injection of $2 \cdot 10^9$ heat-killed cells of *B. pertussis*, phase I, not absorbed (Behringwerke Marburg), in 0.5 ml saline. The 30 mice of group III were

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Table I. Effect of *Bordetella pertussis* and cyclophosphamide on the production of plaque-forming spleen cells in NMRI mice

Group	Treatment on day of immunization	No. of mice[a]	Average number of plaque-forming cells per 10^6 spleen cells at different intervals after immunization:						
			2 days (44 h)	3 days	4 days	5 days	7 days	14 days	21 days
I	$4 \cdot 10^6$ SRBC[b]	3	0.3	27.8	193.4	317.8	94.0	45.5	2.2
II	$4 \cdot 10^6$ SRBC, and $2 \cdot 10^9$ *B. pertussis*	3	31.1	191.5	400.9	890.4	165.1	149.2	55.9
III	$4 \cdot 10^6$ SRBC $2 \cdot 10^9$ *B. pertussis*, and 4 mg cyclophosphamide	3	1.1	3.4	4.5	0	0	20.4	116.8

[a] No. of mice used each day of investigation, [b] sheep red blood cells.

Table II. Effect of *Bordetella pertussis* on the relative spleen weights and the number of plaque-forming spleen cells in NMRI mice[a]

Interval after immunization	No. of mice in the groups					Average relative spleen weights in the groups[b]					Average No. of plaque-forming cells per 10^6 spleen cells in the groups				
	I	II	III	C_1	C_2	I	II	III (mg)	C_1	C_2	I	II	III	C_1	C_2
24 h	7	7	3	–	–	54.9	64.0	63.5	–	–	2.5	3.1	1.4	–	–
30 h	4	4	–	–	–	65.0	66.1	–	–	–	6.2	29.3	–	–	–
44 h	3	3	3	–	–	44.1	72.9	74.2	–	–	40.8	300.8	12.5	–	–
3 days	3	3	3	–	–	58.9	97.1	69.4	–	–	228.8	1009.4	26.1	–	–
4 days	3	2	–	3	–	55.0	103.3	–	67.4	–	2336.2	4716.2	–	Ø	–
5 days	3	2	3	–	3	70.7	96.7	81.1	–	53.7	2027.3	4399.4	12.4	–	1.2
14 days	3	3	3	–	–	60.0	93.6	122.3	–	–	74.6	162.6	2.2	–	–
21 days	3	3	–	–	–	53.4	81.3	–	–	–	36.9	623.1	–	–	–
28 days	4	5	–	–	–	55.5	53.4	–	–	–	11.0	77.0	–	–	–
35 days	5	6	–	–	–	74.2	48.6	–	–	–	4.3	53.1	–	–	–

[a] Treatment of mice. Group I: $8 \cdot 10^8$ sheep red blood cells i.p. Group II: $8 \cdot 10^8$ sheep red blood cells and $2 \cdot 10^9$ *B. pertussis* simultaneously i.p. Group III: $2 \cdot 10^9$ *B. pertussis* i.p. Group C_1: $8 \cdot 10^8$ sheep red blood cells i.p., but spleen cell suspensions of these mice were added to agar containing human erythrocytes, blood group B. Group C_2: normal mice. [b] Relative spleen weight = mg wet spleen/10 g body weight.

immunized in the same manner as the animals of group II, but 2 h before sensitization each mouse was treated by an i.p. injection of 4 mg cyclophosphamide in 0.5 ml saline. 3 mice out of each group, respectively, were sacrificed at various intervals after sensitization and their spleens removed aseptically. The results given in Table I show that already 44 h after sensitization remarkable numbers of plaque-forming spleen cells were demonstrable in pertussis-treated mice (group II) and that in the same group, on all days of investigation, the plaque-forming cells were increased compared to those found in the spleens of the mice of group I. Finally, there was a marked prolongation of antibody production in the spleens of pertussis-treated mice (group II). On the other hand, as indicated in Table I, formation of plaque-forming spleen cells was completely depressed during the first 7 days after immunization by a single dose of 4 mg cyclophosphamide, followed by a slow formation rate of plaque-forming cells in the next 2 weeks. It may be mentioned in this connection that a single dose of 4 mg cyclophosphamide is able to depress completely the susceptibility to systemic anaphylaxis and the formation of circulating antibodies, induced with the aid of pertussis organisms[21].

In order to investigate the adjuvant effect of *B. pertussis* over a longer period of time, and to prove the influence of *B. pertussis* cells on the relative spleen weight (mg spleen/10 g body weight) during the time of investigation, a second experiment was undertaken using NMRI/Han. mice (22–25 g) which were divided in 3 groups. Both the mice-groups I and II were immunized by an i.p. injection of $8 \cdot 10^8$ SRBC. The mice of group II had simultaneously received an additional i.p. injection of $2 \cdot 10^9$ *B. pertussis* cells, while the mice of group III only were treated with the pertussis vaccine. As additional controls, 2 groups of 3 mice each were used: control C_1 (mice immunized in the same manner as the animals of group I, but spleen suspensions of which prepared 4 days after immunization were added to agar containing $4 \cdot 10^8$ human erythrocytes of blood group B) and C_2 (normal mice).

Results presented in Table II show that already 30 h after immunization a distinct plaque formation could be demonstrated with spleen cells of the immunized and pertussis-treated mice (group II), while at this time antibody formation was absent in the spleens of mice of group I. 14 h later the average number of plaque-forming spleen cells found in the mice of group II was about $7^1/_2$ times higher than that demonstrated in the animals of group I. In a markedly lesser degree, short term proliferation of plaque-forming spleen cells was also demonstrable in mice treated solely with pertussis organisms (group III). The multiplication of plaque-forming spleen cells by *B. pertussis* could be demonstrated at all intervals after immunization. Thus the prolongating effect on antibody formation seems to be characteristic not only for anorganic adsorbents and oil phases but also for pertussis organisms. The adjuvant effect of *B. pertussis* cannot be related – at least not alone – to its contents of endotoxin, since the adjuvant effect of endotoxins of gramnegative bacteria is only related to the enhancement but not to prolongation of antibody production.

Our results indicate that the essential mechanism of the action of *B. pertussis* as an immunological adjuvant is connected with its stimulatory influence on the proliferation of immunologically competent cells resulting in an accelerated, increased and prolongated formation of antibody forming cells[22].

Zusammenfassung. Der Wirkungsmechanismus immunologischer Adjuvantien ist nicht bekannt. Mittels Anwendung der Jerneschen Technik wurde daher die Frage untersucht, ob Pertussisorganismen ihre adjuvante Wirksamkeit über eine Vermehrung immunologisch kompetenter Zellen entfalten. Es konnte gezeigt werden, dass die simultane Injektion von *B. pertussis* und Schaferythrocyten im Vergleich zur alleinigen Injektion von Schaferythrocyten bei NMRI-Mäusen zu einer beschleunigten, gesteigerten und verlängerten Bildung antikörperbildender Milzzellen führt.

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