cycles/min at 37 °C for 2 h. $^2/_{10}$ ml of 1 N H_2SO_4 was injected into the aliquot after the 2-h-incubation period. An additional 20 min incubation followed and then the $^{14}CO_2$ was transferred to a vial which contained 10 ml of scintillation fluid (2.5-diphenyloxazole, 4 g and 1.4-*bis*-2-(5 phenyloxazolyl) benzene 0.1 g/l of toluene). The $^{14}CO_2$ was counted in an Aloka liquid scintillation counter (Nihon Musen Co., Tokyo) and the dpm were computed from the measured efficiency of the counter. The percent of the initial radioactivity of the ^{14}C-U-glucose released as $^{14}CO_2$ was calculated.

Experiment 2. The red cell suspensions, solutions A and B, were prepared and mixed and incubated as mentioned in experiment 1. The red cell suspensions, ^{14}C-U-glucose solution, and 0.1 ml of 0.05% methylene blue solution were placed in the flask. The methylene blue was added in an effort to activate glucose monophosphate shunt in the erythrocyte. It has been known that methylene blue stimulates the mechanism of oxydation of hexose-6-phosphate in the erythrocyte by carrying electrons directly to oxygen, thereby permitting the glucose oxidation pathway to recycle[5]. Processes afterwards were the same as those in experiment 1.

The results of the experiments are presented in the Table. As can be seen from the Table, the glycolytic activities of the erythrocytes, expressed as the rates of production of $^{14}CO_2$ from ^{14}C-U-glucose, did not alter in the presence of bilirubin either when the erythrocyte-bilirubin mixture was preincubated ($p > 0.05$) or when it was not preincubated ($p > 0.05$). Since the results were against the expectation that bilirubin may interfere with the glycolytic activities of the erythrocytes, it was felt that further study in the presence of methylene blue would be necessary before offering an explanation for the results. The addition of methylene blue resulted in the increase of glycolytic activities of the erythrocytes by approximately 20 times, confirming the earlier work of BRIN and YONEMOTO[8]. When bilirubin was added to the erythrocyte-methylene blue system, however, the increase of glycolytic activities was markedly impaired. The difference between the 2 was statistically significant both when erythrocyte-bilirubin mixture was preincubated ($p < 0.01$) and when it was not preincubated ($p < 0.01$). Methylene blue has been known to increase the activities of hexose monophosphate shunt pathway in the erythrocytes[8]. Probably an increased reoxidation of the NADPH in the presence of methylene blue will regenerate the reactions of this pathway[9]. Our findings, therefore, suggest that bilirubin might interfere with the transfer of electrons from NADPH to methylene blue and in turn depress the glycolytic activities of the erythrocytes. This interference of bilirubin may be a factor in the recently observed shortened red cell survivals noted in erythrocytes preincubated in the solution containing unconjugated bilirubin[3].

Zusammenfassung. Es wird nachgewiesen, dass die $^{14}CO_2$-Herstellung aus ^{14}C-U-Glukose durch rote Blutkörperchen in Anwesenheit von Methylenblau sich um das etwa 20fache vermehrt. Der Einfluss von Methylenblau wurde auffallend herabgesetzt, wenn Bilirubin dem Erythrozytensystem mit Methylenblau zugesetzt wurde.

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Increased Primary Immune Response and Priming in Mice to Subimmunogenic Doses of Sheep Erythrocytes by *Bordetella pertussis*

The injection of killed cells of *Bordetella pertussis* into mice, alone or together with a second antigen, results in a long-term increase of the wet and dry spleen weights, characterized by a doubling of the cell number and an increased protein synthesis of the individual cell[1]. In this multiplication, besides other proliferating cell systems, antibody – forming cells participate[2,3]. The present study was performed in order to investigate, at the cellular and humoral level, whether the primary immune reaction and the process of priming for the secondary response are altered by *B. pertussis*, if subimmunogenic doses of sheep erythrocytes are used as an antigenic stimulus.

Adult male mice of the inbred strain NMRI weighing 19–26 g were immunized i.p. with 4×10^6 sheep erythrocytes (SE) (group I). In preliminary studies this dose had been determined as the threshold dose allowing the development of a measurable immune response at the cellular level. A second group of mice (group II) was immunized by the simultaneous i.p. injection of 4×10^6 SE and 3×10^9 cells of *B. pertussis* (phase I, not absorbed and killed by treatment with merthiolate for 30 min at 60 °C). A second i.p. injection of 4×10^6 SE was given into the mice of both groups 37 days after the primary antigenic stimulus. At different intervals after the primary and secondary immunization, 5 mice out of each group and 2 animals of the corresponding controls were

The influence of *Bordetella pertussis* (BP) on the development of direct and indirect plaque-forming spleen-cells to a subimmunogenic dose of sheep erythrocytes (SE) in mice

Days after primary immunization	Average numbers of PFC/10^6 spleen cells in mice immunized with 4×10^6 SE		4×10^6 SE + 3×10^9 BP	
	Direct PFC	Indirect PFC	Direct PFC	Indirect PFC
2	1	0	17	0
3	1	0	103	0
4	10	0	122	0
5	31	8	382	509
7	3	0	414	314
10	8	27	169	781
13	2	2	97	939
37	3	3	24	145
40	1	1	26	164
42	4	4	17	82
44	5	0	31	208

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sacrificed, their spleens removed aseptically and their sera collected and pooled. For the quantitative determination of plaque-forming spleen cells (PFC), the direct[4] and indirect[5] agar plaque technique were employed. Suspensions of spleen cells in agarose (Behring-Werke Marburg, Germany) were poured out onto Oxoid No. 3 agar/DEAE-dextran underlayers in petri dishes as described elsewhere[6]. Total serum hemolysin activity of pooled serum samples and those fractions of hemolysins resistant to treatment with 0.125 *M* 2-mercaptoethanol (2-ME) were determined spectrophotometrically at 530 nm according to the 50% hemolysis method[7]. The lowest value considered significant was found to be 10 HU (50% hemolysis units/ml serum)[8]. Total and 2-ME-resistant agglutinating titres of the pooled sera were assayed by standard methods.

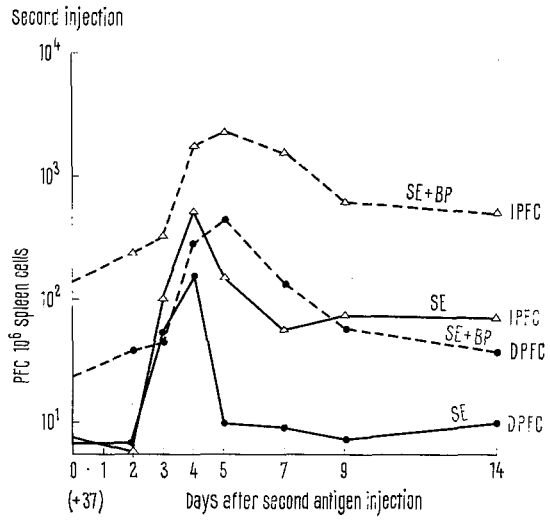

Fig. 1. Effect of an initial injection of killed *B. pertussis* (BP) cells on the appearance of plaque-forming spleen cells after the secondary injection of a subimmunogenic dose of sheep erythrocytes (SE). DPFC, direct plaque-forming cells; IPFC, indirect plaque-forming cells.

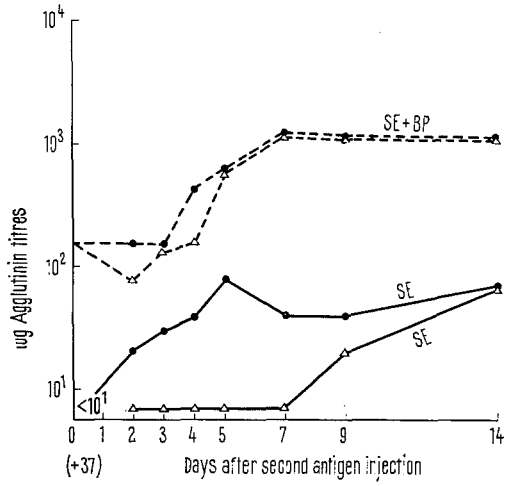

Fig. 2. Effect of an initial injection of killed *B. pertussis* (BP) cells on the agglutinin titres after the secondary injection of a subimmunogenic dose of sheep erythrocytes (SE). ●, total agglutinin activity; △, fractions of the total agglutinin activity resistant to 2-mercaptoethanol.

In the spleens of mice immunized with 4×10^6 SE (group I) small numbers of direct and indirect PFC were found during the primary immune response (Table). Noteworthy amounts of serum hemolysins ($\gtrsim$10 HU) and agglutinins in titres of 1:10 or higher were not demonstrable during the primary immune reaction. This poor response was markedly increased by the additional injection of 3×10^9 cells of *B. pertussis* (Table). Taking into consideration that the strong and long-term elevation of the spleen weights after the injection of killed *B. pertussis* cells is caused in part by a doubling of the cell number[1], this suggests a higher value of the total number of antibody-producing as well as of memory cells. Furthermore the peak value of total hemolysins amounted to 93 HU at day 7. These antibodies were sensitive to treatment with 2-ME. 7S hemolysins, however, first became detectable at day 10 (10 HU) increasing to a value of 33 HU at day 37. Agglutinins appeared at day 10 reaching the peak titre (1:160) at day 37. These agglutinins were resistant to treatment with 2-ME.

When group I was boosted by 4×10^6 SE 37 days after the primary immunization, a typical secondary response was observed, characterized by the prevailing appearance of indirect PFC (Figure 1). In the mice additionally treated with killed cells of *B. pertussis* (group II) the secondary injection of 4×10^6 SE resulted in a markedly increased secondary response at the cellular level (Figure 1). The peak value of 7S hemolysins determined in the sera of group I 9 days after secondary immunization, was 18 HU. The corresponding peak value in the sera of mice initially immunized together with killed cells of *B. pertussis* amounted to 462 HU at day 9. With respect to the agglutinin titres, similar differences were noted (Figure 2).

The results obtained suggest that the additional injection of killed cells of *B. pertussis* not only increases the primary response to subimmunogenic doses of sheep erythrocytes, but also the priming for the secondary response. These findings also provide further evidence of the importance of the use of killed *B. pertussis* cells in certain types of mixed vaccines.

Zusammenfassung. Die i.p. Injektion von 4×10^6 Schaferythrozyten führt bei Mäusen zu einer auf zellulärer Ebene gerade noch messbaren primären Immunitätsreaktion, während humorale Antikörper nicht nachgewiesen werden können. Die zusätzliche Injektion von abgetöteten Keuchhustenbakterien bewirkt nicht nur eine verstärkte Primärreaktion, sondern sie begründet auch eine gesteigerte anamnestische Reaktion, was mit der vermehrten Bildung von «Gedächtniszellen» erklärt wird.

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