Further Studies Pertaining to a *B. pertussis* Factor Inhibiting the Tuberculin Reaction

It has been shown earlier that pertussis vaccine inhibits markedly the tuberculin reaction in BCG-sensitized guinea-pigs[1]. Injection of the vaccine shortly before the tuberculin challenge reduced the reaction intensity by 60–70%.

In these experiments whole pertussis vaccine was used, namely a commercial preparation (pure Bordet-Gengou vaccine) distributed by the Schweizerisches Serum- und Impfinstitut Bern for human use. It contained approximately $40 \cdot 10^9$ cells/ml.

The work recorded here was directed towards a further characterization of the anti-inflammatory *B. pertussis* factor.

Methods. The tuberculin reactions were elicited in guinea-pigs of both sexes weighing 250–500 g. They had been sensitized in a hind-foot pad with living lyophylized BCG vaccine (BCG sec Berna). The sensitizing dose consisted of about $0.2 \cdot 10^6$ cells in 0.1 ml 80% Freund's complete adjuvant. The challenging dose (750 IU tuberculin, Albumosefreies Tuberkulin Berna) was injected in 0.1 ml saline into the depilated skin of both flanks. The ensuing reaction was evaluated after 8 and 24 h by measuring the skin thickness over the reaction centre as previously described[2,3]. For each animal the mean value of the bilateral reactions was tabulated.

The commercial pertussis vaccine used contained as conservants 0.01% merthiolate (sodium ethylmercurithiosalicylate) and 0.072% formaldehyde. Its filtrate is designated as filtrate A. In the experiments 5, 6, 7 and 8, undertaken to test the enzyme sensitivity of the active factor, a vaccine preparation without merthiolate and formaldehyde, but otherwise identical, was utilized[4]. The control groups in the experiments 1–4 received 0.1 mg/ml merthiolate and 0.72 mg/ml formaldehyde in saline, the controls in the experiments 5–8 received saline without these agents.

To test its effect on the tuberculin reaction, the pertussis vaccine was administered twice; namely, the first dose 30 min before and the second dose 8 h after the tuberculin injection. All the vaccine injections were performed intraperitoneally. The experimental groups consisted of 6 or more guinea-pigs, except in experiment 6 where 4 animals per group were used. The average % increase in skin thickness for each experimental group was tabulated and related to the respective control group.

Methodical details (see Tables). Experiment 1: First, it was investigated if the active factor was cell bound or also present in the suspending medium. For this purpose, the vaccine was centrifuged at 23,000 *g* at 4 °C for 7 min. The supernatant was filtered through a millipore filter with 0.22 ± 0.02 μ pore size. Gram staining of the filtrate revealed the complete absence of cells. For group 3, the sediment, consisting of the *B. pertussis* cells, was resuspended in saline. Experiment 2: For groups 7 and 8, centrifugation and resuspension was repeated twice (sediment II) and three times (sediment III). Experiment 3: The cell-free filtrate A, obtained by filtration through the millipore filter, was heated as indicated in Table I. Experiment 4: 30 ml aliquots of the filtrate A were dialysed twice for 8 h against 6 l 0.9% NaCl. Experiment 5: Probes of filtrate B and of saline for the control groups were incubated with papain (Difco, N F VIII) or trypsin (Difco, 1:250) for 1 h at concentrations of 1 mg/ml. The aliquots were exposed to papain at 52 °C and pH 6.5, to trypsin at 40 °C and pH 8. Experiment 6: Probes of filtrate B and of saline for the control groups were incubated with deoxyribonuclease (Fluka) or ribonuclease (Fluka) for 1 h at concentrations of 1 mg/ml. The aliquots were exposed to deoxyribonuclease at 37 °C and pH 6.5 with 0.01 *M* $MgCl_2$ and to ribonuclease at 37 °C and pH 7.5. Experiment 7: To filtrate B and to saline, 1 mg/ml of lysozyme (Fluka, from eggs, 17,000 U/mg) was added with subsequent exposition for 1 h at 37 °C. Experiment 8: To filtrate B and to saline, 1 mg/ml hyaluronidase (Fluka, from bovine testes, lyophylized) was added and exposed for 1 h at 37 °C.

[1] G. L. Floersheim, Int. Archs Allergy appl. Immun. *26*, 340 (1965).
[2] G. L. Floersheim, Helv. physiol. pharmac. Acta *22*, 92 (1964).
[3] G. L. Floersheim, Z. naturw. med. Grundlagenforsch. *2*, 307 (1965).
[4] By courtesy of the Schweizerisches Serum- und Impfinstitut, Bern.

Table I. Effect of filtration, heat and dialysis on the anti-inflammatory activity of *B. pertussis* vaccine

Experiment	Group	Weeks after sensitization	Treatment (i.p.)	% increase in skin thickness (± SD)[a]		Relative difference to controls[b] (%)	
				8 h	24 h	8 h	24 h
1	1	6	Controls, 0.2 ml/100 g saline	24 ± 9	57 ± 9		
	2	6	Vaccine, 0.2 ml/100 g[c]	16 ± 6	40 ± 9	− 33	*− 29*
	3	6	Sediment I, 0.2 ml/100 g[d]	19 ± 9	37 ± 22	− 21	*− 35*
	4	6	Filtrate A, 0.2 ml/100 g	9 ± 9	40 ± 12	*− 62*	*− 31*
2	5	7	Controls, 0.2 ml/100 g saline	43 ± 6	89 ± 11		
	6	7	Sediment I, 0.2 ml/100 g[d]	17 ± 9	50 ± 16	*− 60*	*− 44*
	7	7	Sediment II, 0.2 ml/100 g[d]	27 ± 10	61 ± 6	*− 38*	*− 31*
	8	7	Sediment III, 0.2 ml/100 g[d]	25 ± 13	56 ± 12	*− 41*	*− 37*
3	9	9	Controls, 0.2 ml/100 g saline	27 ± 15	49 ± 12		
	10	9	Filtrate A, 0.2 ml/100 g	6 ± 5	29 ± 10	*− 78*	*− 40*
	11	9	Filtrate A, 0.2 ml/100 g, 30 min at 75 °C	11 ± 10	25 ± 14	*− 61*	*− 50*
	12	9	Filtrate A, 0.2 ml/100 g, 30 min at 90 °C	5 ± 7	18 ± 7	*− 81*	*− 63*
4	13	11	Controls, 0.2 ml/100 g saline	21 ± 6	36 ± 11		
	14	11	Filtrate A, 0.2 ml/100 g	4 ± 6	18 ± 7	*− 88*	*− 50*
	15	11	Filtrate A, 0.2 ml/100 g, dialysed	7 ± 7	22 ± 8	*− 67*	*− 39*

Table II. Effect of enzymes on the anti-inflammatory activity of *B. pertussis* vaccine

Experiment	Group	Weeks after sensitization	Treatment (i.p.)	% increase in skin thickness (± SD)[a]		Relative difference to controls[b] (%)	
				8 h	24 h	8 h	24 h
5	16	3	Controls, 0.4 ml/100 g saline	47 ± 7	84 ± 11		
	17	3	Controls, saline + papain, 0.4 ml/100 g	39 ± 16	75 ± 31	− 17[e]	− 11
	18	3	Controls, saline + trypsin, 0.4 ml/100 g	53 ± 20	91 ± 23	+ 14[e]	+ 9
	19	3	Filtrate A, 0.4 ml/100 g	20 ± 17	48 ± 20	*− 58*[e]	*− 43*
	20	3	Filtrate B, 0.4 ml/100 g	18 ± 15	63 ± 23	*− 60*[e]	*− 26*
	21	3	Filtrate B + papain, 0.4 ml/100 g	28 ± 12	45 ± 24	*− 40*[f]	*− 47*
	22	3	Filtrate B + trypsin, 0.4 ml/100 g	13 ± 5	38 ± 17	*− 71*[g]	*− 54*
6[l]	23	4	Controls, 0.4 ml/100 g saline	49 ± 14	95 ± 15		
	24	4	Controls, saline + deoxyribonuclease, 0.4 ml/100 g	53 ± 11	99 ± 20	+ 8[h]	+ 4
	25	4	Controls, saline + ribonuclease, 0.4 ml/100 g	42 ± 17	95 ± 21	− 14[h]	0
	26	4	Filtrate B, 0.4 ml/100 g	19 ± 12	76 ± 22	*− 61*[h]	− 20
	27	4	Filtrate B + deoxyribonuclease, 0.4 ml/100 g	18 ± 12	76 ± 33	*− 63*[i]	− 20
	28	4	Filtrate B + ribonuclease, 0.4 ml/100 g	21 ± 11	72 ± 21	*− 57*[k]	− 24
7[l]	29	4	Controls, saline + lysozyme, 0.4 ml/100 g	34 ± 19	85 ± 25		
	30	4	Filtrate B + lysozyme, 0.4 ml/100 g	22 ± 12	58 ± 22	*− 35*	*− 32*
8[l]	31	4	Controls, saline + hyaluronidase, 0.4 ml/100 g	74 ± 20	122 ± 27		
	32	4	Filtrate B, 0.4 ml/100 g	50 ± 11	96 ± 21	*− 32*	− 21
	33	4	Filtrate B + hyaluronidase, 0.4 ml/100 g	49 ± 18	97 ± 20	*− 34*	− 21

Tables I and II. [a] SD = standard deviation. [b] Reaction of controls considered as 100%. [c] Containing approximately $8 \cdot 10^9$ cells/0.2 ml. [d] Resuspended in saline, approximately $8 \cdot 10^9$ cells/0.2 ml. [e] Relative difference to control group 16. [f] Relative difference to control group 17. [g] Relative difference to control group 18. [h] Relative difference to control group 23. [i] Relative difference to control group 24. [k] Relative difference to control group 25. [l] The guinea-pigs in the experiments 6, 7 and 8 were treated only once 30 min before the tuberculin injection. Entries in italics relate to data with $p < 0.05$.

Results. They are presented in Tables I and II. The whole vaccine, the filtrate and the resuspended cell sediment were equally active and reduced the skin thickness after 24 h by about 30% (experiment 1). Repeated washings of the sediment led only to a negligible loss of activity (groups 7 and 8). Heating of the filtrate to 75 and 90 °C (groups 11 and 12) did not destroy its activity. Furthermore, the property of the filtrate to inhibit the tuberculin reaction was not altered by dialysis (group 15). The active factor in the *B. pertussis* vaccine filtrate appeared, under the reported conditions, to be resistant to digestion by papain, trypsin, deoxyribonuclease, ribonuclease, lysozyme and hyaluronidase.

According to these findings, the active factor seems to be released by maybe lysed *B. pertussis* cells into the suspending medium. It appears to be water soluble and not dialysable; it does not seem neither to be a protein nor a nucleic acid.

Discussion. Various pharmacological properties have been related to substances from *B. pertussis*. No general theory of their mode of action has yet been presented. The clue to the inhibitory effect of a *B. pertussis* substance on the tuberculin reaction can be traced back to clinical observations. Yet, the marked hematological changes occurring after the administration of pertussis vaccine to experimental animals[5] may be of some importance with regard to its effect on the tuberculin reaction. Thus, the observed depopulation of lymphoid structures might well alter the functional disponibility of immunologically competent cells which initiate or sustain the tuberculin reaction. Of course, it should be noted that the changes of cellular distribution could not be elicited by the injection of the suspending medium but only by the bacterial cells.

The results reported here allow some suggestions regarding the chemical nature of the anti-inflammatory *B. pertussis* factor. Clearly, the activity of the vaccine is not connected only to the cell structure. Several washings of the cells did not significantly affect their capacity to release the active factor. As it is not dialysable, its molecular weight may be expected to be higher than about 1000. Furthermore, by its heat stability and resistance to proteolytic enzymes, the substance does not appear to be proteinic. Work attempting its purification is in progress.

Zusammenfassung. Pertussis Vaccine führt zu einer Hemmung der Tuberkulin-Reaktion bei Meerschweinchen. Der anti-inflammatorisch aktive Faktor wurde näher charakterisiert. Er wird von den Bakterien in das Suspensionsmedium abgegeben und ist filtrierbar, hitzeresistent und nicht dialysierbar. Er blieb widerstandsfähig gegenüber der Einwirkung von Papain, Trypsin, Desoxyribonuklease, Ribonuklease, Lysozym und Hyaluronidase.

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[5] S. I. MORSE, J. exp. Med. *121*, 49 (1965).