Suppression of adjuvant disease by bacterial extracellular products

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The intradermal injection of a suspension of heat-killed mycobacteria in oil (Freund's adjuvant) produces in the rat a generalized disease, adjuvant arthritis or adjuvant disease (Pearson and Wood, 1959). This can be transferred to syngeneic recipients with lymphoid cells from adjuvant-injected donors but not with serum (Waksman and Wennersten, 1963; Pearson and Wood, 1964a; Quagliata and Phillips-Quagliata, 1972) and is therefore considered to be a manifestation of delayed-type or cellular hypersensitivity to disseminated component(s) of the mycobacterium (Quagliata and Phillips-Quagliata, 1972). In addition, the inflammatory reaction at the site of adjuvant injection provides a useful experimental model for the study of inflammation (Perper, Alvarez, Schroder, and Colombo, 1971).

Antilymphocyte serum (ALS), among its many immunosuppressive properties, has been shown to suppress adjuvant disease (Currey and Ziff, 1968). Another property of ALS is that it stimulates the transformation of lymphocytes into large blast-like cells capable of mitosis (Woodruff, Reid, and James, 1967). Streptococcal culture filtrates have recently been shown to have a similar effect on lymphocytes (Taranta, Cuppari, and Quagliata, 1969; Taranta and Cuppari, 1970); we therefore thought it might be of interest to investigate the effect of such filtrates on adjuvant disease. Cellular components of Gram-negative bacteria have previously been shown to prevent adjuvant disease if injected in incomplete adjuvant, but not in saline, before the eliciting adjuvant injection (Wood and Pearson, 1962).

We report that some preparations of Gram-positive as well as Gram-negative bacterial extracellular products injected subcutaneously (s.c.) in saline starting the day before the adjuvant injection markedly suppress adjuvant disease.

Material and methods

Male Fisher 344 rats (215–230 g) were obtained from Microbiological Associates, Bethesda, Md, and maintained on a standard pellet and water ration *ad libitum*.

Adjuvant disease was induced as previously described (Quagliata, Sanders, and Gardner, 1969).

All the rats were weighed immediately before the first injection of the various preparations used and then again as indicated. Bacterial and control preparations were given subcutaneously in 1 ml. doses.

The inflammatory reaction in the paws was assessed according to a commonly accepted 4-point scale as previously described (Quagliata and others, 1969).

Haematocrit, haemoglobin, and red and white blood cell counts, were determined by standard methods.

Differential WBC counts were done after staining with McNeil tetrachrome stain.

The bacterial strains used were *Strep. pyogenes* (Group A beta-haemolytic streptococcus) M-type 3, T-type 29 (Strain F. Hernandez 7/8/69) originally cultured in this laboratory from a child with pharyngitis; *Staph. aureus* 4423 No. 75, *S. typhimurium* No. 25, and *Strep. pneumoniae* R. 36 NC No. 39, obtained from the Department of Microbiology (N.Y.U. School of Medicine).

Each microbial strain was grown overnight in 500 ml. of Todd-Hewitt (TH) broth (Difco Labs., Detroit, Michigan), which were then inoculated into 4.5 l. of pre-warmed TH broth. After 6 hours' incubation the cultures were centrifuged at 4°C. for 30 min. at 7,970 G. An aliquot of each bacterial culture was sterilized by filtration through a Nalgene filter (pores 0.2 μ) and tested for mitogenic activity undiluted and in 1/30, 1/900, 1/27,000, and 1/810,000 dilutions on cultures of human peripheral blood lymphocytes; the mitogenic activity was assayed morphologically as previously described (Taranta and others, 1969). The *Strep. pyogenes* filtrate caused 50 per cent. transformation at an interpolated dilution of 1:500. None of the other bacterial filtrates induced a transformation of 50 per cent. or greater at any of the dilutions tested

(Taranta and Cuppari, unpublished observation). The supernatants and the uninoculated TH broth used as a control were concentrated by pervaporation at room temperature for 18 hrs, dialysed against cold tap-water for 6 hrs and against cold distilled water for 42 hrs. The dialysed materials were then re-concentrated by pervaporation at room temperature to a volume of 100 ml. and again dialysed in the cold against phosphate-buffered saline for 42 hrs. They were then centrifuged at 7,970 G. for 20 min. and filtered through Nalgene filters, after which cultures were negative.

Human serum was obtained from two commercial blood donors. 5× crystallized ovalbumin (Pentex, Inc., Kankakee, Illinois) was prepared as a 30 mg./ml. solution.

The protein content of the preparations was determined as tyrosine residues by the Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951) using human gamma globulins (Cohn fraction II) as a standard. The

Results

FIRST EXPERIMENT

We tested the effect of two schedules of injections of *Strep. pyogenes* preparation A on the development of adjuvant disease. Thirty rats were divided into three groups of ten each. The control group was treated with twenty daily injections of 1 ml. sterile pyrogen-free 0.9 per cent. sodium chloride (saline), starting the day preceding the adjuvant injection. A second group was injected for the same period with 1 ml. of the *Strep. pyogenes* preparation and a third group was injected with the same preparation for 10 days, starting 4 days after the adjuvant injection. The inflammation score for the three groups was recorded until the 90th day after the adjuvant injection, as indicated in Fig. 1.

FIG. 1 Inflammation score for the three uninjected paws (solid lines) and for adjuvant-injected hind paw (broken lines) in the saline-treated control group (open circles) and in the groups treated with the *Strep. pyogenes* preparation for 10 days, starting 4 days after the adjuvant injection (closed triangles), and for 20 days, starting the day before the adjuvant injection (closed circles).

content was as follows:

For <i>Strep. pyogenes</i> preparation A (used for Experiment 1)	Not done
For <i>Strep. pyogenes</i> preparation B (used for Experiment 2)	19.2 mg./ml.
For <i>Strep. pyogenes</i> preparation C (used for Experiment 3)	19.4 mg./ml.
For Todd-Hewitt broth, uninoculated	16.0 mg./ml.
For <i>Staph. aureus</i> preparation	13.8 mg./ml.
For <i>S. typhimurium</i> preparation	18.2 mg./ml.
For <i>Strep. pneumoniae</i> preparation	23.2 mg./ml.

All these preparations were stored in 5-ml. aliquots at $-70^{\circ}\text{C}.$ until use.

Sheep red blood cells (SRBC) were obtained from the City of New York, Department of Health, Bureau of Laboratories, Otisville Branch, washed three times in sterile saline, and re-suspended to a 10 per cent. suspension for immunization and to a 1 per cent. suspension for haemagglutination determinations, which were carried out as described (Levine and Levytska, 1967).

The swelling of the adjuvant-injected paw reached a maximum the day after the injection and remained at that level throughout the period of observation in the control group and in the group receiving the *Strep. pyogenes* preparation starting on Day 4. By contrast, the swelling in the group that received the *Strep. pyogenes* preparation starting the day before the adjuvant injection was markedly reduced at first, and reached control values only by Day 18. The reaction in the three uninjected paws was markedly suppressed in both *Strep. pyogenes*-treated groups, but more so in the group treated for a longer time. In this as in the following experiments there was little variation in the severity of the arthritis among the individual rats of each group. Only the saline-injected rats developed, to a varying degree, the extra-articular manifestations of the disease such as blepharitis, urethritis, and subcutaneous nodules. The *Strep. pyogenes*-treated rats

Table I *Haematological evaluation after a 10- and a 20-day course of Strep. pyogenes preparation (first experiment)**

Blood cell count	After 10 days		After 20 days	
	Saline	Strep. pyogenes	Saline	Strep. pyogenes
Red	$5.47 (\pm 0.19) \times 10^6$	$6.65 (\pm 0.66) \times 10^6$	$4.38 (\pm 0.31) \times 10^6$	$5.01 (\pm 0.23) \times 10^6$
White	$28.5 (\pm 5.59) \times 10^3$	$22.4 (\pm 2.85) \times 10^3$	$27.9 (\pm 5.89) \times 10^3$	$20.2 (\pm 3.40) \times 10^3$

* Mean values ($\pm$ S.D.)

appeared more active, heavier, and generally healthier throughout the period of observation. The marked leucocytosis and anaemia seen in the controls were considerably reduced in the *Strep. pyogenes*-treated rats (Table I).

SECOND EXPERIMENT

This was performed to confirm the results of the first with a newly made *Strep. pyogenes* preparation (preparation B). Ten rats were treated for 20 days, starting the day preceding the adjuvant injection, and ten control rats were concomitantly injected with saline. The joints were scored as before; in addition, weights were also recorded at regular intervals. Photographs of a representative rat of each group are shown in Fig. 2.

As can be seen in Fig. 3 (opposite), preparation B was at least as effective as preparation A both in delaying the onset of swelling in the injected paw and in suppressing arthritis in the uninjected paws.

Although there was an initial weight loss in both groups, a second phase of weight loss occurred only in the controls, coincident with the appearance of arthritis.

WBC, RBC, and differential counts, determined 21 and 48 days after the adjuvant injection, are given in Table II (opposite). On Day 21 the controls had marked leucocytosis and moderate anaemia as compared to the rats injected with the *Strep. pyogenes* preparation. By Day 48 the RBC count of the controls had returned to normal, while that of *Strep. pyogenes*-

treated rats was slightly lower. The WBC count had decreased in the controls and in the *Strep. pyogenes*-treated rats—but was definitely lower in the latter. The differential counts were very similar in both groups.

As in the previous experiments, extra-articular manifestations appeared in the controls but not in the rats injected with the *Strep. pyogenes* preparation, and the general state of health appeared to be better in the latter, as the weight curve clearly shows (Fig. 3).

THIRD EXPERIMENT

This was designed first to exclude the possibility that the suppression of the disease was merely due to the medium in which the streptococci had been grown, or to antigenic competition, and secondly to investigate the specificity of the phenomenon in terms of the bacterial species involved.

37 rats were divided into eight groups and injected daily for 20 days, starting the day before the adjuvant injection, as follows:

- Group 1: Saline.
- Group 2: Todd-Hewitt preparation.
- Group 3: Human serum.
- Group 4: Crystalline ovalbumin.
- Group 5: *Strep. pyogenes* preparation C.
- Group 6: *Strep. pneumoniae* preparation.
- Group 7: *Staph. aureus* preparation.
- Group 8: *S. typhimurium* preparation.

15 days after the adjuvant the rats received 1 ml. SRBC intraperitoneally; they were then bled for

FIG. 2 *Hind paws of a Strep. pyogenes treated (No. 1) and a saline treated (No. 17) rat at, from left to right, 14, 21, 31, 45, and 60 days after adjuvant injection.*

FIG. 3 Weight (triangles, above) and inflammation score (circles, below) in saline-treated (open symbols) and *Strep. pyogenes*-treated (closed symbols) groups. Inflammation score: solid lines for the three uninjected paws, broken lines for the adjuvant-injected paw.

Table II Haematological evaluation 21 and 48 days after adjuvant injection (second experiment)*

Days after adjuvant injection	21		48	
Treatment	Saline	<i>Strep. pyogenes</i>	Saline	<i>Strep. pyogenes</i>
Red blood cell count	$5.77 (\pm 0.88) \times 10^6$	$8.15 (\pm 1.13) \times 10^6$	$8.33 (\pm 1.42) \times 10^6$	$8.07 (\pm 0.81) \times 10^6$
White blood cell count	$37.5 (\pm 4.54) \times 10^3$	$22.6 (\pm 8.52) \times 10^3$	$20.5 (\pm 3.89) \times 10^3$	$14.3 (\pm 4.49) \times 10^3$
Differential:				
Mononuclears	39.8 $\pm$ 8.79	61.7 $\pm$ 8.66	49.7 $\pm$ 6.64	49.1 $\pm$ 7.50
Polys:				
Neutrophils	60.1 $\pm$ 8.62	37.5 $\pm$ 8.52	50.0 $\pm$ 6.53	50.1 $\pm$ 7.17
Eosinophils	0.1 $\pm$ 0.45	0.8 $\pm$ 1.24	0.3 $\pm$ 0.47	0.8 $\pm$ 0.74

* Mean values $\pm$ S.D.

antibody titre determinations 1 and 7 weeks later. Fig. 4 (overleaf) shows the result of this experiment.

In the groups receiving injections of saline or non-bacterial preparations, the swelling of the adjuvant injected paw followed the pattern previously observed in the controls. The swelling of the adjuvant-injected paw was initially decreased in each of the groups of rats receiving preparations of bacterial filtrates. This early local reaction was reduced most markedly in

the groups injected with the *Strep. pyogenes* and with the *S. typhimurium* preparations.

The arthritis in the three uninjected paws was suppressed to a variable extent by the preparations used. Suppression and delay were greatest with the *S. typhimurium* and the *Strep. pyogenes* preparations, while the *Staph. aureus* preparations appeared ineffective. This last preparation caused crusty necrotic lesions at the site of injection. A moderate

FIG. 4 Weight (hatched areas—top of each panel) and inflammation score (bottom) for the various preparations used for treatment; shaded areas represent score for three un.injected paws, and the line the score for the adjuvant injected paw.

reduction of the arthritis was achieved also with the non-bacterial preparations.

The weight loss was practically the same in the saline, *Staph. aureus*, and ovalbumin groups; it was less in the Todd-Hewitt and *Strep. pneumoniae* groups, and much less in the *Strep. pyogenes* and human serum groups. The rats injected with *S. typhimurium* preparation, after a sharp initial drop, started gaining weight at an unusually rapid rate.

Haematological observations are shown in Table III (opposite). The saline-treated group was modera-

tely anaemic both on Day 21 and Day 49. The other groups were less anaemic, the *Strep. pyogenes* group being the most normal in this respect.

The WBC counts showed leucocytosis in the saline-injected group and marked leucopenia in the *S. typhimurium* group on Day 21; the other groups showed intermediate values. On Day 48, while most groups had normal values, the *S. typhimurium* group was still definitely leucopenic.

The differential counts showed that the increase in WBC was due mostly to an increase in neutrophils,

Table III Haematological evaluation 21 and 49 days after adjuvant injection (third experiment)*

Day		Saline	Todd-Hewitt	Human serum	Cryst. ovalbumin	Strep. pyogenes	Strep. pneum.	Staph. aureus	S. typhim.
21	Hct	39.0 ±2.50	48.1 ±2.09	39.5 ±5.18	43.2 ±3.26	48.6 ±3.06	46.0 ±0.94	44.3 ±1.20	42.9 ±1.51
	Hb	12.0 ±0.38	14.5 ±0.20	12.8 ±1.6	14 ±1.18	14.7 ±0.68	14.7 ±0.44	13.7 ±0.29	13.8 ±0.55
	RBC†	6.59 × 10 ⁶ ±0.27	7.35 × 10 ⁶ ±0.67	6.5 × 10 ⁶ ±0.44	7.11 ± 10 ⁶ ±0.80	7.8 × 10 ⁶ ±1.02	6.89 × 10 ⁶ ±1.50	6.89 × 10 ⁶ ±0.70	6.48 × 10 ⁶ ±0.58
	WBC†	31.2 × 10 ³ ±5.41	19 × 10 ³ ±8.34	15.25 ± 10 ³ ±2.16	23.75 × 10 ³ ±5.80	23 × 10 ³ ±1.22	14.8 × 10 ³ ±1.72	27 ± 10 ³ ±4.14	8.25 × 10 ³ ±0.82
	Neut.	67.2 ±7.6	52.0 ±1.63	52.5 ±6.10	61.0 ±12.88	50.25 ±15.28	55.0 ±7.23	61.0 ±17.53	47.75 ±7.75
	Eosin.	0.8 ±0.63	1.0 ±0.81	2.0 ±2.44	1.5 ±0.86	0.25 ±0.43	0.8 ±0.74	1.4 ±1.49	1.25 ±1.29
	Mono.	32.0 ±7.04	47.0 ±1.41	45.5 ±4.27	37.5 ±12.11	49.5 ±15.58	44.2 ±7.08	37.6 ±17.36	51.0 ±9.02
49	Hct	40.6 ±3.47	43.8 ±9.07	49.5 ±2.12	41.0 ±6.43	47.0 ±2.50	44.5 ±6.41	45.6 ±6.17	47.6 ±0.73
	Hb	11.3 ±1.96	12.8 ±1.62	14.3 ±0.46	11.4 ±2.09	13.6 ±1.09	12.0 ±1.88	13.2 ±1.33	12.9 ±0.72
	RBC†	6.8 × 10 ⁶ ±0.59	7.1 × 10 ⁶ ±0.32	8.3 × 10 ⁶ ±0.16	6.7 × 10 ⁶ ±0.54	8.2 × 10 ⁶ ±0.28	7.0 × 10 ⁶ ±0.83	7.9 ± 10 ⁶ ±0.50	7.3 × 10 ⁶ ±0.53
	WBC†	19 × 10 ³ ±5.43	16 × 10 ³ ±4.32	21.5 × 10 ³ ±3.04	36 × 10 ³ ±14.50	14 × 10 ³ ±2.44	13 × 10 ³ ±0.81	21.2 × 10 ³ ±4.16	7 × 10 ³ ±1.87
	Neut.	34.0 ±7.78	45.66 ±5.43	49.75 ±13.40	59.5 ±6.87	37.0 ±6.08	47.0 ±6.48	33.6 ±10.44	47.0 ±8.54
	Eosin.	2.0 ±0.81	1.0 ±0.70	2.25 ±1.29	0.75 ±0.43	1.75 ±1.92	2.33 ±0.94	1.2 ±1.16	0.25 ±0.43
	Mono.	64.0 ±8.52	53.3 ±4.71	48.0 ±3.24	39.75 ±8.95	61.25 ±7.56	50.66 ±5.90	65.5 ±11.67	52.75 ±8.16

* Mean values (±S.D.).

† Pre-treatment values were: RBC=8.1 × 10⁶; WBC=13.8 × 10³.

which was particularly marked at Day 21 in the saline, ovalbumin, and *Staph. aureus* groups and at Day 49 in the ovalbumin group.

Table IV summarizes the haemagglutination data. One week after immunization with SRBC, the only significant reduction in the anti-SRBC titre, as compared with the saline-treated controls, was observed in the group treated with the *S. typhimurium*

Table IV Agglutination titres* against sheep red blood cells in rats treated with preparations of bacterial extracellular products or control preparations

20 days treatment with	Time after immunization with sheep red blood cells†	
	1 week	6 weeks
Saline	12.72 ± 1.62	11.98 ± 1.69
Todd-Hewitt	12.32 ± 0.81	10.98 ± 1.24
Human serum	10.82 ± 1.11	12.07 ± 2.16
Crystalline ovalbumin	13.57 ± 1.29	15.32 ± 0.00
<i>Strep. pyogenes</i>	12.82 ± 0.86	12.92 ± 1.20
<i>Strep. pneumoniae</i>	12.52 ± 0.74	12.32 ± 1.00
<i>Staph. aureus</i>	11.52 ± 2.31	11.52 ± 1.72
<i>S. typhimurium</i>	9.57 ± 0.82‡	11.82 ± 0.50

* Mean log₂ titre ± standard deviation.

† All rats were immunized with sheep red blood cells 2 weeks after the injection of adjuvant and the beginning of treatment with the various preparations.

‡ Significantly lower than saline-injected control group (P<0.05), Student's t-test.

preparation. 6 weeks after immunization no significant differences were observed.

Discussion

The development of adjuvant disease presumably depends on the integrity of both immunological and inflammatory mechanisms. Predominantly anti-inflammatory drugs, such as cortisone, suppress the manifestations of the disease for as long as they are administered, but shortly after their administration is stopped the disease manifests itself (Newbould, 1963). Immunosuppressive agents, on the other hand, may suppress the disease permanently (reviewed by Quagliata and others, 1969). The local reaction which occurs soon after the adjuvant injection depends mainly on inflammatory mechanisms (Perper and others, 1971). The suppression of adjuvant disease, particularly with the *Strep. pyogenes* and *S. typhimurium* preparations, appeared to affect both inflammatory and immune responses: the local early reaction at the site of the adjuvant injection was decreased, and the arthritis in the uninjected paws was not only delayed but suppressed, i.e. its intensity was decreased if and when it appeared.

It is probable that the mechanisms involved in the suppression of adjuvant arthritis are not the same for

the preparations of extracellular products of Gram-positive and Gram-negative bacteria. Only the rats injected with the Gram-negative bacterial preparation developed a marked leucopenia (see Table III); this could well have interfered with the inflammatory and the immune response.

That micro-organisms may produce substances which interfere with the inflammatory response is not, of course, a novel idea. Levaditi (1918) may have been the first to report an *in vitro* correlate of this interference—the ability of some streptococcal strains to kill leucocytes when engulfed by them. With a few exceptions (Malakian and Schwab, 1968, 1971; Hanna and Watson, 1968), the ability of micro-organisms to interfere with immune mechanisms does not appear to have been studied, although both streptococci and staphylococci are known to produce substances capable of reacting with human lymphocytes (Taranta and others, 1969; Ling, Spicer, James, and Williamson, 1965). Immunosuppression with a streptococcal preparation which differs from ours in being of cellular rather than extracellular origin (Malakian and Schwab, 1968, 1971) has been reported. Immunosuppression with preparations of streptococcal pyrogenic exotoxin (Hanna and Watson, 1968) has also been reported. Whether this toxin is the mediator of the effect we have described remains to be determined as far as the *Strep. pyogenes* preparations are concerned. It cannot, of course, be the mediator of the effect observed with the *Strep. pneumoniae* or *S. typhimurium* preparations, since neither of these organisms is known to produce streptococcal pyrogenic exotoxin or any toxin closely related to it.

The possibility that the inhibitory effect of the *Strep. pyogenes* preparations might be due to the streptococcal mitogen known to be present in them (Taranta and others, 1969; Taranta and Cuppari, 1970) deserves consideration, since other substances capable of inducing lymphocyte transformation, notably ALS, are immunosuppressive. Again, however, the arthritis inhibitory effect of the *Strep. pneumoniae* and *S. typhimurium* preparations cannot be explained on this basis, since these species are known not to produce extracellular substances comparable to the streptococcal and staphylococcal

mitogens (Taranta and Cuppari, 1970) and the particular strains tested were not exceptions (Taranta and Cuppari, unpublished observations).

The possibility that the suppression of adjuvant disease by extracellular bacterial products is the result of antigenic competition (Wood and Pearson, 1962; Gery and Waksman, 1967; Pearson and Wood, 1964b) was considered: therefore two additional controls were included in the third experiment, one (crystalline ovalbumin) consisting essentially of a single protein and the other (human serum) consisting of about as many proteins as are present in the *Strep. pyogenes* culture filtrate. Neither of these control preparations provided a reduction of the disease of the degree obtained with the active bacterial products—thus failing to support the hypothesis of antigenic competition as the only or main mechanism of the suppression of adjuvant disease obtained with the bacterial products.

It should be noted that the maximum suppression obtained (in *S. typhimurium* and *Strep. pyogenes*-treated rats) was quantitatively similar to that reported by others and by one of us with ALS and other powerful disruptors of immune mechanisms (Currey and Ziff, 1968; Quagliata and others, 1969; Quagliata, Phillips-Quagliata, and Floersheim, 1972; Zurier and Quagliata, 1971). Therefore, further study of this suppression and definition of the extracellular bacterial products involved may be rewarding in terms of experimental pathology and therapeutics.

Summary

Both the inflammatory response at the site of adjuvant injection and the subsequent development of adjuvant disease in the rat were markedly suppressed by the administration of extracellular products from some bacterial species. *Salmonella typhimurium* products caused marked leucopenia, inhibition of circulating antibody response, and suppression of adjuvant disease. *Strep. pyogenes* products did not cause leucopenia or inhibition of circulating antibody response, yet they caused marked inhibition of adjuvant disease. *Strep. pneumoniae* products had similar, but less marked effects, while *Staph. aureus* products were ineffective.

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