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DEPRESSION OF DELAYED HYPERSENSITIVITY BY PRETREATMENT WITH FREUND-TYPE ADJUVANTS

I. DESCRIPTION OF THE PHENOMENON

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SUMMARY

Delayed hypersensitivity induced by antigen in Freund's complete adjuvant (FCA) can be depressed by pretreatment with FCA alone beforehand. In the guinea-pig, pretreatment with FCA depressed the 24 hr skin reactions which otherwise followed immunization with bovine γ-globulin, human serum albumin and the arsanil-N-acetyl tyrosine in FCA. The depression ranged from 25 to 75% and 4 hr skin reactions were also depressed. In contrast, haemolytic and cytophilic antibody to BGG was not depressed by pretreatment with FCA before immunization with antigen in FCA.

It was not necessary to use the same adjuvant for pretreatment and immunization, and pretreatment with FCA or *Corynebacterium parvum* adjuvant depressed delayed hypersensitivity to BGG induced by BGG in either FCA or *C. parvum* adjuvant.

In both the guinea-pig and rat, pretreatment with FCA, and soluble antigen acted synergically in depressing 24 hr delayed skin reactions. Thus pretreatment with either FCA or soluble antigen, before immunization with antigen in FCA depressed delayed hypersensitivity skin reactions, and pretreatment with both agents caused a greater depression than either singly. However pretreatment with FCA did not alter the depression of antibody production caused by soluble antigen.

In the mouse, pretreatment with FCA or *C. parvum* depressed contact sensitivity to picryl chloride induced by picryl chloride in FCA. Contact sensitivity to picryl chloride and oxazolone induced by skin painting was not depressed by pretreatment with FCA.

INTRODUCTION

Immunization with antigen in Freund's complete adjuvant (FCA) gives rise to strong delayed

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hypersensitivity. In contrast pretreatment with FCA alone depresses the delayed hypersensitivity which otherwise follows immunization with antigen in FCA (Jankovic, 1962; Lisak & Kies, 1968). It also blocks the induction of autoimmune disease by brain in FCA in the guinea-pig (Kies & Alvord, 1958). This paper confirms and extends the findings of Jankovic (1962) that pretreatment with FCA depresses delayed hypersensitivity.

Pretreatment with soluble antigen is also able to depress the delayed hypersensitivity which follows immunization with antigen in FCA (Dvorak *et al.*, 1965). This paper shows that pretreatment with FCA, followed by soluble antigen causes a greater depression of delayed hypersentitivity skin reactions than either singly.

MATERIALS AND METHODS

Animals

Outbred Dunkin-Hartley guinea-pigs, and albino rats were obtained from commercial breeders. Inbred CBA mice were obtained from the Transplantation Immunology Unit, The London Hospital.

All the animals in any one experiment were of the same age and sex, and were randomly allocated to experimental groups.

Bacterial adjuvants

Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were made as described in Davey & Asherson (1967). Human heat-killed tubercle bacilli were used. *Corynebacterium parvum* (heat-killed: a gift from Dr G. Biozzi) and *Bordetella pertussis* (Per/Vac: Burroughs–Wellcome) were added to the saline phase of FIA. Intravenous injections were made in saline.

Antigens

Bovine γ-globulin (Fraction V, Armour Pharmaceuticals) and human serum albumin (Behringwerke) were dissolved in saline just before use. Picryl chloride was obtained from Hopkin & Williams, and oxazolone (2-phenyl-4-ethoxymethylene oxazolone) from British Drug Houses. They were dissolved in absolute ethanol for sensitization, and in olive oil for ear testing. Arsanil-N-acetyl tyrosine was prepared according to Jones & Leskowitz (1965).

Immunization and adjuvant pretreatment

Pretreatment with bacterial adjuvant. Guinea-pigs and rats were pretreated with Freund-type bacterial adjuvant in three footpads 10 days before definitive immunization. Guinea-pigs received a total of 0·15 ml (0·05 ml/footpad) adjuvant containing 1 mg/ml tubercle bacilli or 2 mg/ml *C. parvum;* rats received a total of 0·15 ml adjuvant containing 3 mg/ml tubercle bacilli or 3 mg/ml *C. parvum* or 5×10^{10} *B. pertussis.*

Mice were pretreated either with 0·06 ml Freund-type adjuvant distributed over the front footpads and the base of the tail 10 days before immunization, (3 mg/ml tubercle bacilli or 8 mg/ml *C. parvum* or 2×10^{11} *B. pertussis*/ml), or with a saline suspension of bacteria intravenously (0·7 mg *C. parvum* or $1{\cdot}2 \times 10^{10}$ *B. pertussis*) 4 days before immunization.

Immunization with antigen in adjuvant. Guinea-pigs were immunized with 50 μg bovine γ-globulin (BGG) and/or 100 μg human serum albumin (HSA) in 0·05 ml Freund-type adjuvant (1 mg/ml tubercle bacilli or 2 mg/ml *C. parvum*) in the fourth footpad. Rats were

immunized with 0·5 mg BGG in 0·05 ml FCA (3 mg/ml tubercle bacilli) in the fourth footpad.

Mice were immunized with picryl chloride or oxazolone by mixing 1 volume of a freshly prepared solution of the agent in ethanol with 19 volumes of saline. The suspension was then emulsified immediately in FCA (tubercle bacilli 5 mg/ml final concentration).

Immunization with antigen alone. Mice were immunized by painting 0·1 ml of 3% oxazolone or 7% picryl chloride, in ethanol, on the skin of the freshly clipped abdomen.

Tests for antibody

Haemagglutination was performed according to Stavitsky (1954) but pH 7·2 buffer was used in place of pH 6·4 buffer. Haemolytic antibody was measured by the complement lysis of sheep red cells coupled to antigen with bisdiazobenzidine (Davey, Asherson & Stone, 1971). Cytophilic antibody was measured by the method of Boyden. See Davey & Asherson (1967).

Skin and ear tests

Guinea-pigs were challenged by intradermal injection of 0·1 ml antigen solution in saline. Before injection, the animals were shaved, depilated with 'Buto' (Biometics Ltd) and the injection sites marked with a 'Magic Marker'. Induration, i.e. the double skin thickness, was measured with a dial gauge (System Kroplin; model Quicktest AO2T) before injection, at 24 hr, and sometimes at 4 and 48 hr (Nelson & Boyden, 1964). Erythema was measured with a transparent ruler, in two directions at right angles.

Mice were challenged and the increment of ear thickness measured at 24 hr (Asherson & Ptak, 1969). Rats were challenged by intradermal injection into the ear of 20 μl antigen solution, containing 50 μg BGG, and the increment of ear thickness measured similarly.

In the tables, 1 unit represents 10^{-3} cm in rats and mouse experiments, and 10^{-2} cm in the guinea-pig experiments. The results are expressed as the increment of thickness over the pre-injection reading, which was approximately 53 units in the case of the rat, 27 units in the mouse, and 28 units in the guinea-pig.

Statistics

The two-tail *t*-test was used unless the data were not normally distributed when the two-tail Mann–Whitney *U*-test was used (Siegel, 1956).

RESULTS

Depression of 4 and 24 hr skin reactions by pretreatment with Freund's complete adjuvant

Guinea-pigs were pretreated with FCA, while control guinea-pigs were left untreated. 10 days later, all the animals were immunized with BGG in FCA, and challenged with BGG intradermally after a further 14 days. Table 1 shows that the mean diameter of the 24 hr skin reactions in the control guinea-pigs was 22·2 mm. In contrast, the mean diameter in the guinea-pigs pretreated with FCA was significantly lower, at 18·0 mm.

Table 1 also shows that pretreatment with FCA depressed the 24 hr skin reactions to HSA from 23·2 to 6·0 mm and the reaction to arsanil-N-acetyl tyrosine from 12·6 to 7·1 mm.

It was concluded that pretreatment with FCA depressed 24 hr skin reactions to protein

and synthetic antigens. Table 2 shows that pretreatment with FCA also depressed 4 hr skin reactions to BGG.

Pretreatment with FCA probably failed to depress delayed hypersensitivity early after immunization, and no depression was seen in four experiments in which the guinea-pigs were challenged within 10 days of immunization.

TABLE 1. Depression of 24 hr skin reactions in the guinea-pig by pretreatment with Freund's complete adjuvant

Synoptic table

Exp. no.	Antigen	Mean diameter of 24 hr skin reaction (mm) to test antigen		% Depression
		No pretreatment (control)	Pretreatment with FCA 10 days before BGG in FCA	
1	Bovine γ-globulin	22·2±2·0	18·0±2·6	19*
2	Human serum albumin	23·2±3·7	6·0±4·2	74‡
3	Arsanil-N-acetyl tyrosine	12·6±1·9	7·1±2·7	44†

Guinea-pigs were pretreated with FCA, while other guinea-pigs were left untreated. 10 days later, all the guinea-pigs were immunized in the fourth footpad with BGG, or HSA, or 2×10^{-8} moles (9 μg) arsanil-N-acetyl tyrosine in FCA. The table shows the mean diameter and SD of the erythema 24 hr after intradermal injection of 25 μg of the test antigen (BGG, HSA or arsanil-guinea-pig albumin) at 14 days. There were five guinea-pigs in each group.

* $0{\cdot}01<P<0{\cdot}05$; † $0{\cdot}001<P<0{\cdot}01$; ‡ $P<0{\cdot}001$.

TABLE 2. Effect of pretreatment with Freund's complete adjuvant and Freund's incomplete adjuvant on 4 and 24 hr skin reactions to bovine γ-globulin the guinea-pig

Pretreatment −10 days	No. of animals	Mean skin reactions (mm) to 25 μg BGG at 17 days	
		4 hr induration (10^{-2}cm)	24 hr erythema (mm)
Nil (control)	11	24·0±7·0	20·7±1·7
Freund's incomplete adjuvant	5	22·6±7·0	20·6±2·3
Freund's complete adjuvant	5	13·9±4·0†	14·6±1·4‡

See legend to Table 1.

* $0{\cdot}01<P<0{\cdot}05$; † $0{\cdot}001<P<0{\cdot}01$; ‡ $P<0{\cdot}001$.

Failure of Freund's incomplete adjuvant or silica to depress delayed hypersensitivity

Table 2 shows that pretreatment with FIA did not depress delayed hypersensitivity to BGG. In a separate experiment with six guinea-pigs in each group, pretreatment with

30 mg silica (0·5–1 μ, 3 μ and 3–5 μ diameter) into three footpads failed to depress delayed hypersensitivity to BGG and HSA at 21 days, although in the same experiment guinea-pigs pretreated with FCA showed a depression of 30%.

Depression of delayed hypersensitivity induced by antigen in one adjuvant by pretreatment with a different adjuvant

Table 3 confirms that pretreatment with FCA depressed delayed hypersensitivity induced by antigen in FCA. It also shows that pretreatment with *C. parvum* adjuvant depressed the delayed hypersensitivity induced by antigen in *C. parvum* adjuvant. Moreover, pretreatment with FCA depressed delayed hypersensitivity induced by antigen in *C. parvum* adjuvant, and the converse was true in one out of two experiments.

TABLE 3. Depression of 24 hr skin reactions to bovine γ-globulin in the guinea-pig by pretreatment with Freund's complete adjuvant or *C. parvum* adjuvant

Adjuvant		Mean diameter of erythema at 24 hr (mm)
Pretreatment	Immunization	
Nil	FCA (control)	18·2±2·6
FCA	FCA	13·6±2·8†
CP‡ adjuvant	FCA	16·4±2·6
Nil	CP adjuvant (control)	15·7±6·6
FCA	CP adjuvant	9·4±1·2*
CP adjuvant	CP adjuvant	8·7±3·6*

Each figure is based on five to eight animals. In a second experiment, pretreatment with either FCA or *C. parvum* adjuvant significantly depressed the 24 hr skin reactions by about 25%.

* $0{\cdot}01 < P < 0{\cdot}05$; † $0{\cdot}001 < P < 0{\cdot}01$; ‡ *C. parvum* adjuvant.

Failure of Freund's complete adjuvant to block the depression of delayed hypersensitivity by soluble antigen

Pretreatment with soluble antigen blocks antibody production in the mouse, and both antibody production and delayed hypersensitivity in the guinea-pig. Dresser (1961) showed that pretreatment with FCA blocked the depression of antibody production caused by soluble antigen in the mouse. The following experiments show that pretreatment with FCA does not block the immunodepressive effect of soluble antigen in the guinea-pig. In fact, pretreatment with FCA followed by soluble antigen causes a greater depression of delayed hypersensitivity than pretreatment with either alone.

Guinea-pigs were pretreated with FCA 10 days before immunization, or left untreated. 7 days before immunization, the guinea-pigs received either 1 mg BGG or 1 mg HSA in saline, intravenously. All the guinea-pigs were immunized with a mixture of 50 μg BGG and 100 μg HSA, in FCA on day 0, and skin tests were undertaken 14 days later. Table 4 shows that pretreatment with either FCA or BGG reduced the 24 hr reaction by 21–26%. In

contrast, pretreatment with FCA followed by soluble antigen caused a reduction of 76%. The 24 hr reaction to HSA was virtually abolished by pretreatment with FCA followed by

TABLE 4. Effect of pretreatment with Freund's complete adjuvant followed by soluble antigen upon 24 hr skin reactions and antibody production in the guinea-pig

Pretreatment		Mean 24 hr skin reactions (mm) at 14 days		Mean antibody levels at 20 days	
−10 days	−7 days	25 μg BGG	25 μg HSA	Haemolytic	Cytophilic
Nil	HSA	24·3 ± 3·2	9·7 ± 7·6†	3·5	5·3
Nil	BGG	18·2 ± 5·8*	23·2 ± 3·7	1·2	< 3
FCA	HSA	19·2 ± 3·5*	1·2 ± 1·4‡	6·9*	6·4
FCA	BGG	5·8 ± 4·4‡	6·0 ± 3·0‡	0·9	< 3

There are six guinea-pigs in each group. The antibody titres are in $\log_2$ units, i.e. 1 = ½ dilution. In a comparable experiment, guinea-pigs were immunized with BGG in FCA (instead of a mixture of BGG and HSA). The same pattern of results was obtained, except that there was no significant increase in the haemolytic titre 14 days after immunization in the group pretreated with FCA.

* $0{\cdot}01 < P < 0{\cdot}05$; † $0{\cdot}001 < P < 0{\cdot}01$; ‡ $P < 0{\cdot}001$.

TABLE 5. Depression of 24 hr skin reactions to bovine γ-globulin in the rat by pretreatment with Freund's complete adjuvant, and soluble antigen

Pretreatment	Mean increment of ear thickness (10^{-3} cm) at 24 hr					
	Rats not pretreated with BGG	Rats pretreated with BGG −7 days	*P*	Rats not pretreated with soluble BGG	Rats pretreated with soluble BGG day −7	*P*
Nil (control)	70·2	35·4	< 0·001	78·7	53·7	< 0·001
FCA −21 days	44·3	28·5	N.S.	52·0†	39·3	N.S.
FCA −10 days	52·5*	28·7	< 0·01	46·5†	32·7†	N.S.
FCA −8 days	50·0*	26·8	< 0·001	57·5*	36·2†	< 0·05
FCA −7 days	45·0	28·7	< 0·05	47·5†	25·8‡	< 0·05
FCA −6 days	40·0	30·7	N.S.	54·2†	35·8†	< 0·05
FCA −2 days	49·5*	29·2	< 0·05	71·0	37·3*	< 0·01

Rats were pretreated with FCA in three footpads at the stated times before immunization with BGG in FCA. Some rats also received 5 mg BGG in saline intravenously 7 days before immunization. Antigen was injected intradermally into the ear 14 days after immunization. The table shows the mean increment in ear thickness 24 hr later. The asterisks show the significance of the depression caused by FCA as compared with the nil (control) group which did not receive FCA. The *P* values give the significance of the depression caused by pretreatment with BGG as compared with the groups that did not receive BGG.

* $0{\cdot}01 < P < 0{\cdot}05$; † $0{\cdot}001 < P < 0{\cdot}01$; ‡ $P < 0{\cdot}001$.

soluble antigen. Table 4 also shows that pretreatment with FCA significantly increased haemolytic antibody levels in one out of two experiments.

It was concluded that pretreatment with FCA followed by soluble antigen caused a greater depression of delayed hypersensitivity than either singly.

Table 5 shows similar findings in the rat. FCA depressed delayed hypersensitivity when given 6–21 days before immunization. At −2 days, however, FCA caused no significant depression, although it augmented the depressive effect of soluble BGG. Table 5 also shows that the 4 hr (Arthus-type) reaction was depressed by pretreatment with FCA at −2 to −21 days, and by soluble BGG. However, pretreatment with FCA did not increase the depression caused by soluble BGG.

Depression of delayed hypersensitivity by pretreatment with adjuvant in the rat

Rats were pretreated with FCA, *C. parvum* adjuvant, *B. pertussis* adjuvant, or FIA

TABLE 6. Depression of 4 and 24 hr skin reactions to bovine γ-globulin in the rat by pretreatment with Freund-type adjuvants

Pretreatment −10 days	Increment of ear thickness (10^{-3} cm)	
	4 hr	24 hr
Nil (control)	60·3±15·3	85·2±11·9
Freund's incomplete adjuvant	47·2±8·1	60·3±15·5*
Freund's complete adjuvant	39·4±8·2†	49·7±18·1‡
C. parvum adjuvant	49·2±11·5	54·5±15·1†
B. pertussis adjuvant	67·4±8·5	81·4±10·7

The rats were challenged intradermally 14 days after immunization with BGG in FCA. There were seven rats in each group. The asterisks, dagger and double dagger give the significance of the depression relative to the nil group which was not pretreated.

* $0{\cdot}01 < P < 0{\cdot}05$; † $0{\cdot}001 < P < 0{\cdot}01$; ‡ $P < 0{\cdot}001$.

alone. 10 days later, they were immunized with 1 mg BGG in FCA and challenged 14 days later.

Table 6 shows that FCA significantly depressed 4 and 24 hr skin reactions to BGG. There was also a significant, but smaller effect of FIA. *C. parvum* adjuvant caused no more depression than FIA, while *B. pertussis* adjuvant caused no depression, perhaps because *B. pertussis* increases the action of histamine and other pharmacological agents. These results were confirmed in a second experiment.

It was concluded that pretreatment with FCA, and to a lesser extent, FIA, depressed 4 and 24 hr reactions in the rat.

Depression of contact sensitivity in the mouse

Contact sensitivity to picryl chloride was depressed by pretreatment with FCA, *C. parvum* adjuvant and *B. pertussis* adjuvant, and by *C. parvum* but not *B. pertussis* intravenously (see Table 7).

Contact sensitivity to oxazolone was significantly depressed by pretreatment with *C.*

parvum intravenously at −4 days [the control value was 12·2±2·5 (six mice) as compared with 5·6±2·3 (four mice)]. However, there was no depression by pretreatment with FCA in four separate experiments.

Although as seen above, pretreatment with FCA depressed contact sensitivity induced by picryl chloride in FCA, it had no effect in two experiments on contact sensitivity induced by skin painting with picryl chloride. It also failed to influence contact sensitivity to oxazolone induced by skin painting in three experiments.

TABLE 7. Depression of contact sensitivity to picryl chloride in the mouse by pretreatment with adjuvants

Day	Pretreatment adjuvant	Route	Mean increment in ear thickness at 24 hr (mm)
	Nil (control)		15·0±2·3
−10	Freund's complete adjuvant	Footpad	6·9±1·7‡
−10	*C. parvum* adjuvant	Footpad	8·7±1·9‡
−10	*B. pertussis* adjuvant	Footpad	9·1±1·2‡
−4	*C. parvum* in saline	i.v.	5·5±0·9‡
−4	*B. pertussis* in saline	i.v.	16·8±3·0

After the pretreatment all mice were immunized with 50 μg picryl chloride in FCA. 8 days later, the mice were challenged by painting the ears with a 1% solution of picryl chloride in olive oil. The table shows the increment of ear thickness at 24 hr, expressed as mean ±SD. There were ten mice in the control group and five in the other groups. Asterisks show the significance of the depression relative to the nil (control) group.

‡ $P<0·001$.

DISCUSSION

The results show that pretreatment with Freund's complete adjuvant depressed the response to antigen in FCA, as measured by 4 and 24 hr skin reactions. The phenomenon is general, and occurred with several antigen and in both guinea-pigs and rats, and under some conditions in mice.

The depression of the 24 hr skin reaction is a complex phenomenon and due in part to an anti-inflammatory effect of pretreatment with FCA and in part to a depression of the central state of immunity (Allwood & Asherson, 1971). The phenomenon in the guinea-pig, but not apparently in the rat, requires the bacterial component of the Freund's complete adjuvant. It is not due to the tissue damage or to the secondary response which occurs when an animal pretreated with FCA is subsequently immunized with antigen in FCA. The evidence for this is that the phenomenon occurs in guinea-pigs pretreated with a bacterial adjuvant (FCA) which contains tubercle bacilli and then immunized with antigen in another bacterial adjuvant (*C. parvum* adjuvant).

In the present system pretreatment with FCA depressed skin reactions. However the effect of FCA injected separately from antigen depends upon the test system and the time of the injection. Thus injection of FCA alone may depress or increase resistence to tumours

(Berman, Allison & Pereira, 1967; Goldner, Girardi & Hilleman, 1965). See Asherson & Allwood (1969).

Pretreatment with BGG in saline before immunization with BGG in CFA depressed 24 hr skin reactions in the guinea-pig (Dvorak *et al.*, 1965). We confirmed these findings and showed that pretreatment with FCA followed by BGG in saline further depressed the 24 hr skin reaction. It is known that pretreatment with BGG in saline depresses the central state of immunity as shown by failure of the peritoneal exudate cells of these animals to passively transfer delayed hypersensitivity (Asherson, 1966). It is not known whether the additional effect of pretreatment with FCA is mainly due to its anti-inflammatory action or to its ability to depress the central state of delayed hypersensitivity.

The present findings drew attention to the possibility that bacterial agents may depress delayed hypersensitivity skin reactions in human disease. This may explain the loss of the tuberculin reaction in miliary tuberculosis and of a number of delayed hypersensitivity skin reactions in lepromatous leprosy.

It raises the question whether diminished delayed hypersensitivity skin reactions in immune deficiency disease is sometimes secondary to bacterial infection. Finally it suggests that care is needed in the choice of nonspecific agents such as BGG which are used to increase resistence to tumours in humans.

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