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THE EFFECT OF PERTUSSIS VACCINE ON THE IMMUNE RESPONSE OF MICE TO SHEEP RED BLOOD CELLS

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SUMMARY

Bordetella pertussis is an adjuvant when given to mice immunized with sheep RBC. The adjuvant activity of pertussis is reflected in an increase in the number of cells producing antibody as measured by the localized haemolysis in gel technique. γG-PFC have a more marked response to pertussis than do γM-PFC, and in general γG-PFC are more sensitive than γM to variations in dose or injection schedule of pertussis organisms. Pertussis increases the response to doses of antigen which previously were considered to be maximal. The distribution of PFC between spleen, blood and lymph nodes is altered by pertussis injections.

INTRODUCTION

Bordetella pertussis was shown to have adjuvant properties by Greenberg & Fleming (1947) and Kind (1957). Since then many workers have confirmed these observations (see Munoz, 1964). More recently, Finger, Emmerling & Schmidt (1967) have shown that pertussis organisms (pertussis) will increase the number of mouse spleen cells producing antibody in response to sheep red blood cell (sheep RBC) immunization. They also showed that γG-producing (indirect) PFC are more sensitive than γM-PFC to the adjuvant activity of pertussis (Finger *et al.*, 1968).

Pertussis causes a lymphocytosis (Morse, 1965) and a redistribution of lymphocytes (Morse & Riester, 1967). In the work reported here we examine the effects of different doses of sheep RBC and pertussis and of different injection schedules on the number and distribution of γM- and γG-PFC.

MATERIALS AND METHODS

CBA male mice aged from 3 to 4½ months were used in these experiments. Sheep red blood cells, supplied as whole sheep blood in Alsever's solution, were obtained from Burroughs Wellcome Ltd. Pertussis organisms (*Bordetella pertussis*) were a gift from Glaxo Ltd.

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The pertussis organisms were formalin killed, washed by centrifugation and resuspended in sterile saline containing 0·01% merthiolate, and a concentration of 1×10^{10} organisms per ml.

All injections of sheep RBC and pertussis were made intraperitoneally (i.p.). Injections of pertussis made at the same time as the injection of sheep RBC are designated 'day 0' injections; day -3 and $+3$ are injections made respectively 3 days before and 3 days after the injection of sheep RBC.

The localized haemolysis in gel (LHG) assay was carried out as previously described in detail (Dresser & Wortis, 1967; Wortis, Taylor & Dresser, 1968). Developing sera used were (a) a polyspecific rabbit antiserum with anti-(mouse) Fab activity which was added to the plates after 1 hr of incubation and before complement addition, thereby avoiding detectable inhibition of direct γM-PFC (Wortis, Dresser & Anderson, 1969); (b) a rabbit antiserum prepared against the Fc fragment of myeloma protein 5563 which reacted with both γG_2 subclasses; (c) anti-Ig-la allotype sera prepared in C57Bl/6 mice injected with pertussis coated with CBA anti-pertussis antibody (Dresser & Wortis, 1967). The Ig-la allotype specificity is confined to the γG_{2a} subclass of immunoglobulin (Fahey, Wunderlich & Mishall, 1964); (d) a mixture of rabbit antisera designed to give optimal development of each of the three γG classes (γG_1, γG_{2a} and γG_{2b}). The specificity of all the developing sera was tested as described previously (Wortis *et al.*, 1969).

Spleen cell suspensions were prepared as previously described (Dresser & Wortis, 1967), using a homogenizer made from a loose fitting PTFE pestel in a 15 ml round bottomed centrifuge tube. Lymph nodes were prepared in the same manner, except that the capsules were cut with scissor before homogenization. Blood leucocytes were prepared from buffy coat as follows: mice were injected (i.p.) with 50 i.u. of heparin (Pularin, Evans Medical Ltd). $\frac{1}{2}$–$1\frac{1}{2}$ hr later the mice were anaesthetized by ether, and bled from the heart for 0·8 ml of blood into a 1 ml plastic tuberculin syringe fitted with a 26 g needle. A 12 cm length of polythene cannula was attached to the needle and the blood was gently squirted to the bottom of a piece of polythene tube 13 cm long and sealed at one end, which had been inserted into a 2·5 mm internal diameter and 12·5 cm long glass centrifuge tube. This method of loading allows the tube to be completely filled without any air bubbles. After centrifugation at 1750 *g*, for 10 min (20°C) the polythene tube was removed from the glass supporting tube, laid flat and the tube cut quickly with a clean sharp razor blade 1 mm above the buffy coat. The buffy coat plus about 1 mm of packed erythrocytes was then cut off above a tube containing 5–7 ml of Gey's solution. The cells were suspended immediately and centrifuged at 600 *g*, for 5 min (20°C) in the same way as for spleen and lymph node cells. The supernatant was removed and the cells made up in the minimum amount of Gey's solution necessary both for the LHG assay and for a count of the number of lymphoid cells in the suspension (Coulter, Model B). The cell suspensions were kept in an ice-water bath until plated.

EXPERIMENTS AND RESULTS

The consequences of injecting 20×10^8 *pertussis at the same time as* 4×10^6 *sheep RBC*

This experiment was carried out to investigate the effect of 20×10^8 pertussis, injected i.p., on the response to 4×10^6 sheep RBC injected on the same day. The LHG assay was carried out in the usual manner with polyspecific serum (a) mentioned in the previous section. There were four groups: group 1 was not injected with anything, group 2 was injected with

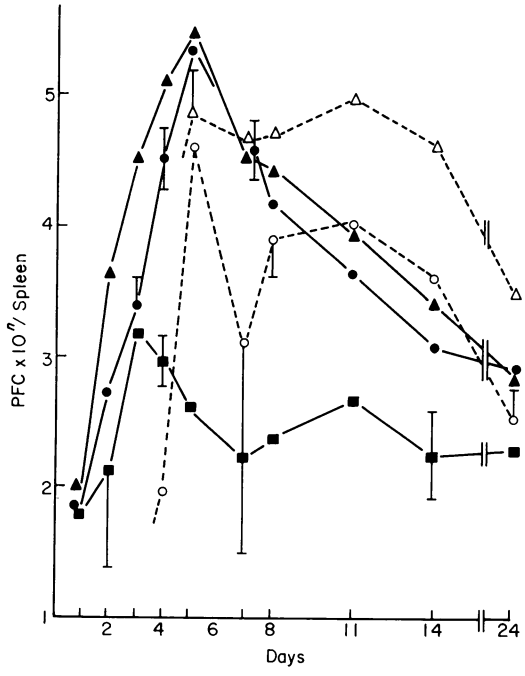

FIG. 1. PFC/spleen in mice receiving 4×10^6 sheep RBC and/or 20×10^8 pertussis: ■, direct PFC after 20×10^8 pertussis; ●, direct PFC after 4×10^6 sheep RBC; ▲, direct PFC after sheep RBC plus pertussis; ○, γG-PFC after sheep RBC; △, γG-PFC after sheep RBC plus pertussis. The largest standard errors are shown.

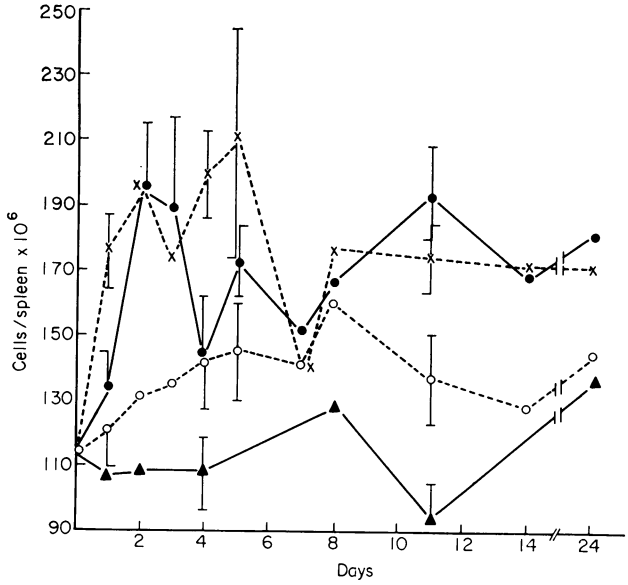

FIG. 2. Nucleated cells/spleen in mice receiving 4×10^6 sheep RBC and/or 20×10^8 pertussis: ▲, untreated mice; ●, pertussis alone; ○ sheep RBC alone; ×, sheep RBC plus pertussis.

sheep RBC alone, group 3 was injected with pertussis alone, while the mice in group 4 received both pertussis and sheep RBC. Four mice from each group were examined for spleen PFC on days 1, 2, 3, 4, 5, 7, 8, 11, 14 and 24 after the injections. The time-response curves are illustrated in Fig. 1. Pertussis itself causes an increase in γM-PFC. The number of γM and γG plaques seen in group 2 (sheep RBC) plus those in group 3 (pertussis) are less than those seen in group 4 (sheep RBC + pertussis).

Fig. 2 illustrates the response to the various injections in terms of the numbers of nucleated cells recovered from the spleens of the mice assayed for PFC. The count was carried out using a Celloscope (A. B. Lars Lindberg & Co., Stockholm, Sweden) with the threshold set to exclude cells with the same or a lesser volume than mouse erythrocytes. It can be seen that in general, pertussis greatly increases the number of nucleated cells in the spleen (cf. Beneke, Finger & Emmerling, 1968; Dresser, 1968) compared with uninjected mice or mice injected with sheep RBC alone.

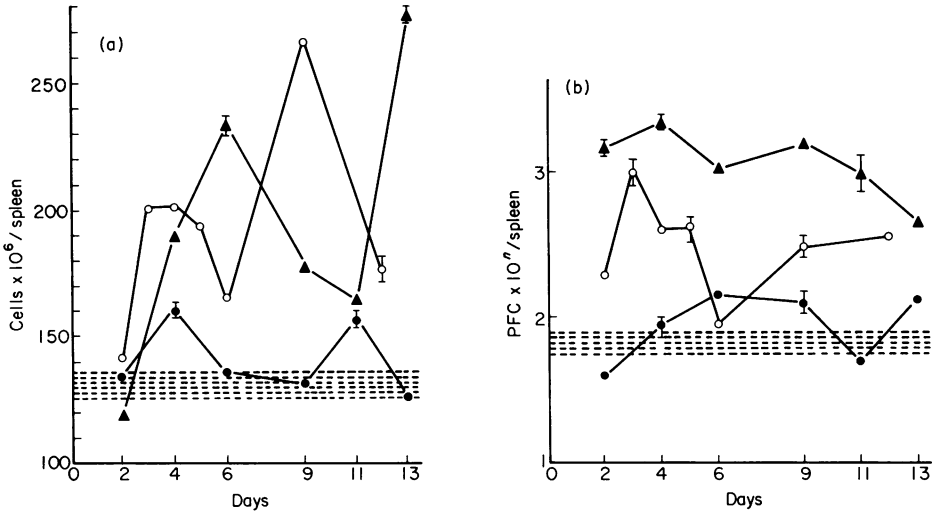

FIG. 3. (a) Nucleated cells/spleen in mice receiving different doses of pertussis. (b) Direct PFC/spleen in mice receiving different doses of pertussis: ●, 4×10^8; ○, 20×10^8; ▲, 100×10^8. Only the largest standard errors are shown. The shaded area represents the value (± one standard error) for several hundred untreated mice examined over a 3-year period.

The effects of pertussis given alone

When pertussis organisms are injected i.p. into CBA mice there is an increase in the nucleated cell population of the spleen and in the total number of direct PFC, but no increase in γG-PFC. The magnitude of both of these responses is related to the number of pertussis organisms injected: 4×10^8 bacteria have a just measurable effect, and 100×10^8 bacteria are more effective than 20×10^8 (Fig. 3a, b). Also, there is an increase in the number of PFC/10^6 spleen cells in each spleen. Hence the increase in direct PFC is not just a reflection of the general increase in cellularity. In contrast, mice given an i.p. injection of 20×10^8 pertussis 8 months after being given 4×10^7 sheep RBC showed no increase in PFC. Prior to the pertussis injection these mice had 1300 direct PFC/spleen: a twenty-fold increase over

the 'background' in uninjected mice. Groups of four mice were examined for PFC on days 1, 2, 3, 4, 5, 8, 11 and 16 after the injection of pertussis, at which times they had respectively 432, 1200, 1600, 1617, 1770, 1500, 824 and 682 PFC/spleen. Except for the day 1 results these figures do not differ significantly from the number of direct PFC found on day 0 (two-tailed t-test on $\log_{10}(x+1)$ transformed counts). It is apparent that 20×10^8 pertussis do not act as a secondary challenge in mice preimmunized with sheep RBC.

The effect of injecting pertussis at different times in relation to the injection of sheep RBC

Mice were immunized with 4×10^7 sheep RBC by i.p. injection. 20×10^8 pertussis organisms were injected on days -5, -3, 0, $+1$, $+3$ and $+5$ in relation to the injection of sheep RBC. Simultaneous (day 0) injection of pertussis results in higher peak numbers of PFC than in any of the other groups (Fig. 4a, b). γG_{2a}-PFC were more sensitive than γM-PFC to

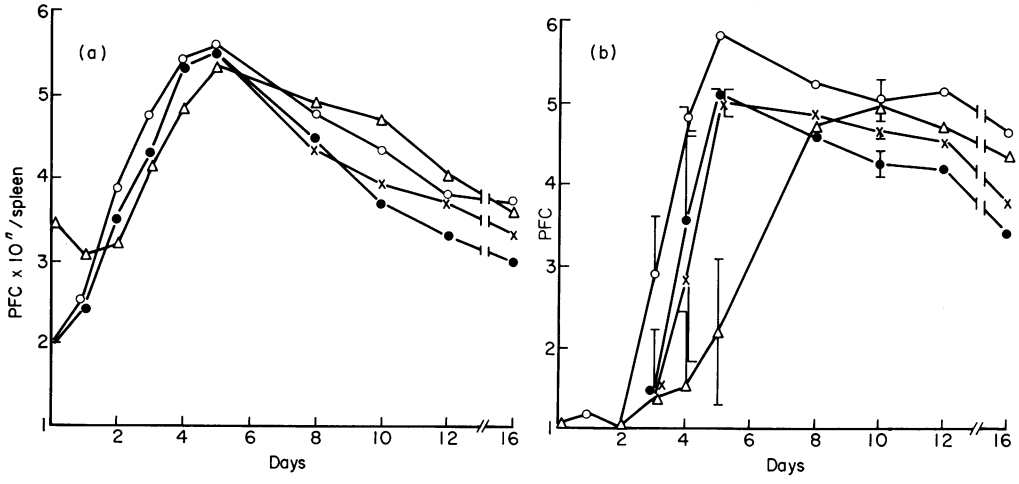

FIG. 4. Direct PFC/spleen (a), and γG_{2a}-PFC/spleen (b), in mice receiving 20×10^8 pertussis 3 days before △; 3 days after × (the same as sheep RBC alone for days 1, 2 and 3); or together with ○, 4×10^7 sheep RBC; ●, sheep RBC alone.

variations in timing of the pertussis injection. For example, simultaneous injection caused an early rise and an elevated peak of γG_{2a}-PFC but an injection of pertussis on day -3 caused a profound depression in the anti-sheep RBC response on days 3, 4 and 5. Integration of time-response curves can provide some information about the total PFC response over an arbitrary unit of time (Wortis *et al.*, 1968). Table 1 lists such integration data (days 0–16) for this experiment. Simultaneous injection of pertussis and sheep RBC produces the greatest augmentation of the splenic PFC count for γM-, γG- and γG_{2a}-PFC. Injection on day -1 produced a slight decrease in the numbers of detected PFC of all three classes.

The effect of pertussis on the response to threshold and supraoptimal doses of sheep RBC

A dose of 4×10^3 sheep RBC injected i.p. produces a direct PFC response which is only just detectable and no increase in numbers of γG-PFC (Wortis *et al.*, 1966). While this small

number of sheep RBC could lack a sufficient number of antigenic units to stimulate a response, it is also possible that 4×10^3 are recognized as antigen, but they lack a sufficient amount of adjuvanticity (Dresser, 1968).

TABLE 1. The ability of 20×10^8 pertussis to act as an adjuvant with respect to sheep RBC depends in part on the relative timing of injections

Group	Day of pertussis injection*	γM (direct)† PFC	γG-PFC†	γG_{2a}-PFC†
1	−5	1105 (120)‡	2070 (169)‡	547 (124)‡
2	−3	890 (97)	2000 (163)	525 (119)
3	−1	769 (84)	1165 (95)	287 (65)
4	0	1346 (146)	3997 (327)	2614 (593)
5	1	869 (94)	2323 (190)	994 (226)
6	3	956 (104)	1425 (130)	595 (135)
7	5	980 (107)	1836 (150)	675 (153)
8	Nil control	920 —	1224 —	440 —

* Sheep RBC injected day 0.

† PFC (days): Integrated time–response curve between days 0 and 16 (for economy of space all values have been divided by 10^3).

‡ The numbers in parenthesis represent mean PFC/spleen as a percentage of the 'Nil control'.

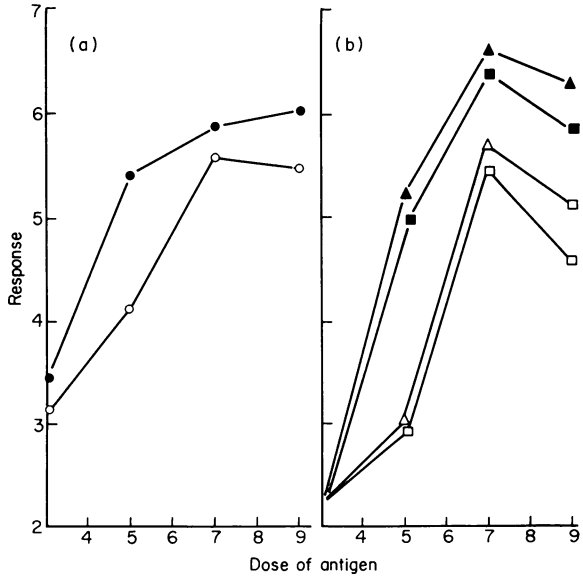

FIG. 5. Response measured by integrating the time response curve (days 2–16) for PFC after 4×10^3, 4×10^5, 4×10^7 and 4×10^9 sheep RBC. Open symbols sheep RBC alone, closed symbols sheep RBC plus pertussis. (a) Direct PFC; (b) △ and ▲, γG_2-PFC, □ and ■, γG_{2a}PFC.

Mice were immunized with 4×10^3, 4×10^5, 4×10^7 or 4×10^9 sheep RBC with or without a simultaneous injection of pertussis; the integrated responses are shown in Fig. 5. 4×10^3 sheep RBC produce a barely detectable increase in the numbers of direct PFC compared with the 'background', while 20×10^8 pertussis alone causes a larger response. The combination of pertussis and 4×10^3 sheep RBC is no more effective than pertussis alone. No γG_2-PFC are found after immunization with this low dose of sheep RBC even when pertussis is used.

At the other extreme, 4×10^9 sheep RBC are known to produce a smaller total PFC response for most classes of PFC than does 4×10^7 or 4×10^8 sheep RBC (Wortis *et al.*, 1969). It is not known whether this plateau represents an absolute limit on the number of

TABLE 2. The adjuvant effect of 100×10^8 pertussis organisms is greater than that of 4×10^8 pertussis. Furthermore, if antigen alone does not produce a γG response, pertussis will have no adjuvant effect in terms of this class of PFC

Class	Dose of sheep RBC	Dose of pertussis: Nil	4×10^8	100×10^8
γM	Nil	1*	1	15
	4×10^4	(4)†	24	43
	1×10^6	(50)†	128	395
	4×10^7	1263	995	1761
	1×10^9	671	750	2473
γG	Nil	<1	<1	<1
	4×10^4	(7)†	7	2
	1×10^6	(35)†	56	1402
	4×10^7	2791	2998	20671
	1×10^9	1093	1766	25293
γG_{2a}	Nil	<1	<1	<1
	4×10^4	(<1)†	<1	1
	1×10^6	(6)†	16	547
	4×10^7	804	940	8320
	1×10^9	294	326	8124

* All the values in this table represent the integrated time response curves between days 2 and 13 (PFC—days $\times 10^{-3}$).

† The numbers in parentheses are mean values derived from several previous experiments; no estimate was made of these values in this experiment.

PFC that can exist in the spleen (due, for instance, to a lack of anatomical space) or to some other homeostatic mechanism such as the regulatory effect of circulating antibody to sheep RBC (cf. Rowley & Fitch, 1964; Wigzell, 1966). The injection of pertussis in addition to high doses of sheep RBC led to an increase in the response resulting from the injection of these maximal doses of sheep RBC without pertussis. This increase in the overall level of the response is not simply a function of a prolonged 'peak' response because the number of PFC found on 'peak' days was also elevated by pertussis.

Fig. 5 also shows that 4×10^9 sheep RBC give a lower total response than 4×10^7 and that

this phenomenon of a decreased response induced by supraoptimal doses of antigen is also seen in the pertussis treated groups.

Two similar experiments confirmed that pertussis could increase the response in terms of γG-PFC but could not initiate it. Thus, if a dose of antigen was too small to stimulate γG_1 or γG_{2a}-PFC, the addition of pertussis would not lead to the appearance of PFC of either of these classes (Tables 2 and 3). The experiment recorded in Table 2 confirmed that pertussis could raise the 'plateau'.

TABLE 3. Plaque forming cells per spleen ($\log_{10}$) on days 4, 6, 8 and 12 after immunization (i p.) by 4×10^3, 4×10^5 and 4×10^7 sheep RBC; half the mice were also injected (i.p.) with 20×10^8 pertussis on day 0

Day	Dose of sheep RBC	γM (direct) PFC		γG_1-PFC		γG_{2a}-PFC	
		No*	Yes*	No	Yes	No	Yes
4	4×10^3	2·2	3·1	0†	1·3	0	1·6
6		2·4	2·5	0	0	1·7	0
8		2·3	2·0	0	0	2·1	1·8
12		1·2	2·3	0	0	1·4	1·6
4	4×10^5	3·1	3·9	0	0	0	2·8
6		3·9	4·4	0	0	3·3	3·1
8		3·5	3·9	0	2·7	0	3·9
12		2·2	3·6	0	0	2·1	2·0
4	4×10^7	4·9	5·2	2·1	5·0	2·2	4·9
6		4·1	4·5	4·3	4·5	4·7	5·3
8		3·2	4·4	0	0	3·4	4·4
12		2·7	3·6	3·0	4·0	3·0	4·5
4	Nil	—‡	3·3	—	0	—	1·0
6		—	2·7	—	0	—	0
8		—	2·2	—	0	—	0
12		—	2·9	—	0	—	0

* No/Yes refers to the i.p. injection of pertussis.

† Zero (0) indicates that no increase in the number of PFC was detected.

‡ 'Background' of γM-PFC 30–80/spleen (~1·5); no background γG_1- or γG_{2a}-PFC have been detected.

In each experiment, the γG-PFC response was increased by pertussis proportionally more than the γM response. This differential effect was apparent in both relative and absolute terms but was not dependent on the dose of sheep RBC.

The relative effectiveness of different doses of pertussis

In several experiments it was found that 100×10^8 pertussis organisms were a more powerful adjuvant than 20×10^8 or 4×10^8 organisms. Integrated time responses from such an experiment are shown in Table 2.

The effect of pertussis on the time of appearance of PFC

Mice were immunized with 4×10^5 or 4×10^7 sheep RBC and half the mice were also

given 20×10^8 pertussis organisms simultaneously. Groups of four mice were subsequently examined for PFC on each day from day 2 to day 10 after injection. The time response curves for anti-sheep RBC antibody producing cells, expressed in terms of PFC of each class, are parallel, irrespective of the dose of sheep RBC and whether or not pertussis was also injected (Fig. 6). Significantly, the γG_{2a} 'peak' following 4×10^5 sheep RBC appears on day 9, either with or without the additional injection of pertussis, and similarly the γG_{2a} 'peak' following 4×10^7 sheep RBC is on day 6. The pertussis appears to affect the magnitude but not the timing of the response. The experiment recorded in Table 3 shows a similar result.

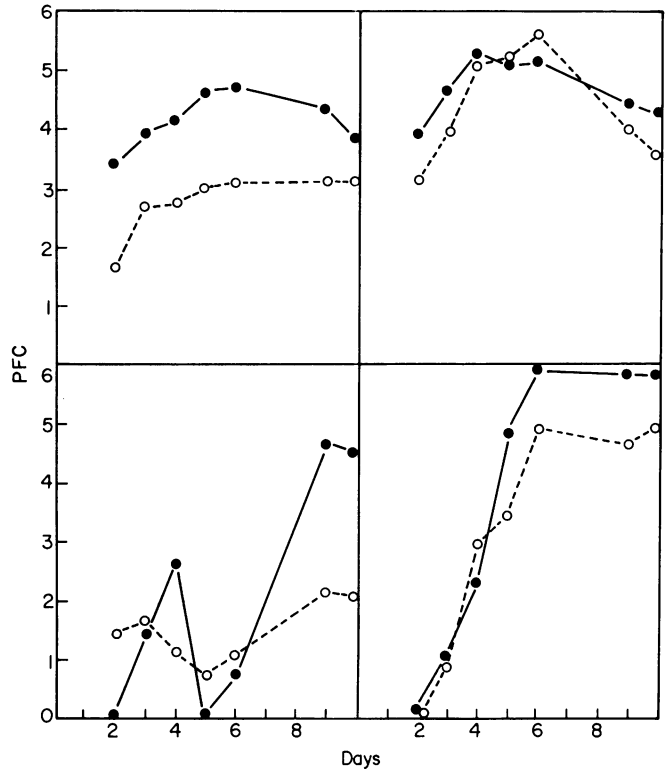

FIG. 6. PFC/spleen in mice receiving 4×10^5 (left) or 4×10^7 (right) sheep RBC i.p. Direct PFC (top) and γG_{2a}PFC (bottom) responses are shown: ○, sheep RBC alone; ●, sheep RBC plus pertussis.

The distribution of PFC after simultaneous i.p. injection of pertussis and sheep RBC

It has been known for some time that after i.p. immunization the main site for the production of antibody to sheep RBC is the spleen (Rowley, 1950; Wissler *et al.*, 1953; Adler, 1965). It therefore seemed relevant to find out if the adjuvant effect of pertussis was confined to the spleen or if it also increased the numbers of PFC in other lymphoid organs.

Fig. 7(a–h) illustrate the rise and fall of γM-, γG_2- and γG_{2a}-PFC in spleens, mesenteric lymph nodes, pooled peripheral (superficial and deep cervical, axillary, brachial, inguinal,

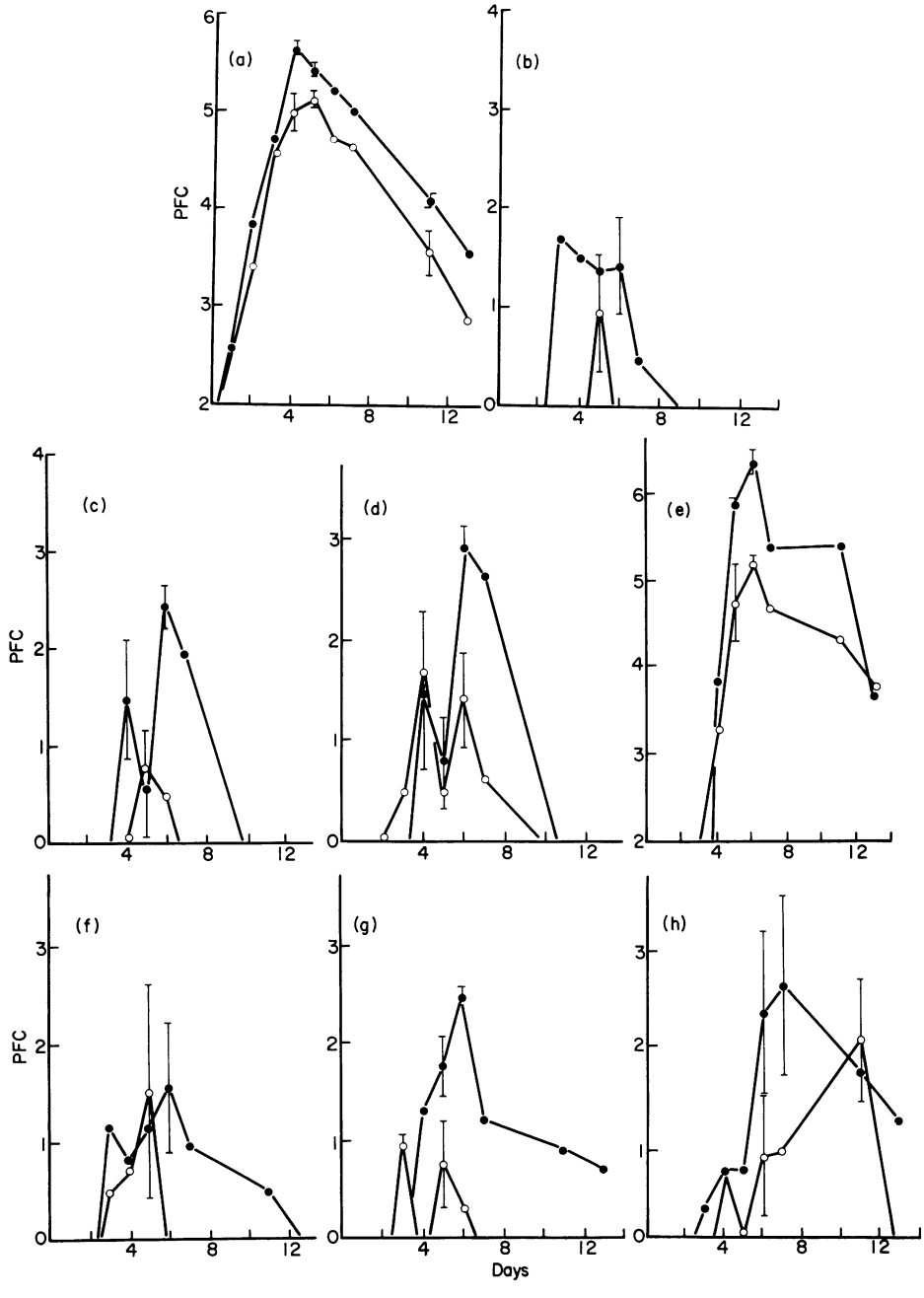

FIG. 7. PFC/organ in mice receiving 4×10^7 sheep RBC i.p. with (●) or without (○) 20×10^8 pertussis. Figures a–d are direct PFC; figures e–h are γG_{2a}PFC. Assays of spleen (a, e), blood (b, f), pooled peripheral lymph nodes (c, g) and mesenteric node (d, h) are shown. The value for PFC/blood is based on the assumption that the total 'leucocyte' blood volume (in ml) of CBA mice is about 12% of the body weight in grams.

para-aortic and popliteal) lymph nodes, and blood leucocytes (buffy coat). The mice in this experiment were all injected with 4×10^7 sheep RBC and half the mice were also injected with 20×10^8 pertussis (day 0).

It is clear that pertussis increases the number of circulating PFC. In view of the low numbers of PFC seen in organs other than the spleen and the degree of uncertainty introduced into these results by the large standard errors, the experiment illustrated in Fig. 7 has been repeated, and the results are presented in Table 4. In this experiment the mice were injected with 4×10^7 sheep RBC and 100×10^8 pertussis (day 0); spleens were not assayed. As might be expected from the results described earlier in this paper, 1×10^{10} pertussis has a much greater effect than 20×10^8 pertussis. The data in Table 4 confirm the previous experiment in showing the early appearance of PFC in the blood followed by the appearance of PFC in the lymph nodes, a process which is either entirely stimulated by pertussis or else enormously increased from that seen in mice injected with SRBC alone.

TABLE 4. Plaque forming cells in blood, peripheral lymph nodes and mesenteric lymph nodes of CBA mice* 4 and 7 days after being immunized (i.p.) by 4×10^7 sheep RBC; half the mice received 20×10^8 pertussis in addition to the sheep RBC

		Day 4				Day 7			
Tissue	Pertussis	Cells† $\times 10^{-6}$	Direct PFC*	γG‡-PFC	γG_{2a}-PFC	Cells $\times 10^{-6}$	Direct PFC	γG-PFC	γG_{2a}-PFC
Blood	No	2·2§	0	0	0	4·8	13	6	18
	Yes	5·3	529	216	3	10·0	435	674	167
Mesenteric	No	26·5	0	1	1	21·9	16	64	28
lymph nodes	Yes	10·7	2	2	0	7·9	156	562	98
Peripheral	No	17·2	2	0	0	18·3	50	117	99
lymph nodes	Yes	18·9	4	1	3	14·4	3598	15,774	224

* The values which are PFC per 'organ' are the geometric means derived from individual assays of six mice.

† For economy of space all cell counts have been divided by 10^6 (No./organ).

‡ γG-PFC were developed using serum (C) (see Materials and Methods).

§ Lymphoid cells in 0·8 ml of whole blood.

DISCUSSION

Pertussis organisms act as an adjuvant when injected into mice concomitantly with an antigen. The non-specific effect of pertussis can be seen in a raised level of circulating antibody to the antigen and, when sheep red blood cells are used as the antigen, it has been shown that the 'adjuvant effect' is manifested as an increased number of antibody-producing cells in addition to a raised level of humoral antibodies (Finger *et al.*, 1967, 1968). In this paper, we have extended the work of Finger and co-workers and have shown that an injection of pertussis made simultaneously with an injection of sheep RBC results in a relatively greater increase in numbers of γG_1- and γG_{2a}-PFC than of γM-PFC. A similar differential class effect has been shown for antibodies to protein antigens (Wilkinson,

Flemming & White, 1967) and as an increase in the level of serum γ-globulin (Barth, McLaughlin & Fahey, 1965), after the injection of adjuvants other than pertussis.

Unless a given dose of antigen is itself large enough to elicit an immune response, an additional injection of pertussis will not help to initiate such a response. It can be seen in Tables 2 and 3 that this is true for each class independently of the others: for instance, 4×10^5 sheep RBC will elicit a γM-PFC response but not a γG_1-PFC response, and a simultaneous injection of 20×10^8 pertussis will increase the γM response but does nothing to induce the appearance of cells producing γG_1 antibody. Nevertheless, 4×10^5 sheep RBC elicit a small γG_{2a}-PFC response and 20×10^8 pertussis has a marked effect in increasing the number of cells making antibody of this class. This result suggests that pertussis does not act by increasing the effective antigen dose. Further evidence which supports this view comes from those experiments where an injection of pertussis resulted in an increased response to a dose of sheep RBC which was at the antigen-dose maximum (Wortis *et al.*, 1969). The latter result suggests that the maximum response is not determined by space limitations or antibody feedback.

Our mice have a 'background' of 66 ± 10 anti-sheep RBC direct-PFC per spleen. It has been concluded that these PFC are γM because they are direct and because they are inhibited by anti-mouse μ-chain and anti-mouse Fab sera (Wortis *et al.*, 1969). An injection of pertussis alone results in a marked increase in the number of anti-sheep RBC γM-PFC. This is not just a non-specific stimulation of cell proliferation, since there is an increase in PFC/10^6 spleen cells.

Mice injected with 4×10^7 sheep RBC 6–8 months previously have a 'background' of 1300 γM-PFC/spleen. However, an injection of pertussis has no effect on this 'background' which suggests the possibility that different kinds of cell are responsible for the different backgrounds. One possible difference is that the background PFC in non-immunized mice are unlike post-immunization PFC (Mitchell & Miller, 1968) in being thymus-derived. This is improbable because a cytotoxic serum specific for thymus-derived cells (θ antigen) failed to reduce (*in vitro*) the number of non-immunized background PFC in a population of spleen cells (M. C. Raff & D. W. Dresser, unpublished experiment). The increase in the non-immunized 'background' could be due to the presence of cross-reacting or shared antigenic determinants. However, we do not think that such shared determinants exist in significant amounts, for the following reasons:

(1) The 4-day γM-PFC 'response' to 20×10^8 pertussis is the same as that elicited by 4×10^5 sheep RBC (Fig. 1; Wortis *et al.*, 1966). The increase in the number of γM-PFC due to the addition of 20×10^8 pertussis to 4×10^7 sheep RBC is far in excess of the increase in numbers expected from an 'equivalent' increase of 4×10^5 in the number of immunizing sheep RBC.

(2) Table 5 shows that mice with a high level of agglutinating antibody for sheep RBC do not have significantly more anti-pertussis agglutinins than are present in normal serum.

(3) 20×10^8 pertussis failed to elicit a secondary response in mice primed 6–8 months previously, as mentioned earlier, whereas an 'equivalent-dose' (4×10^5) of sheep RBC is capable of doing so.

The timing of an injection of pertussis, in relation to the injection of antigen, seems to be important. For example, if the 'adjuvant' had been injected 1 or 3 days before the antigen there was a slight suppression of the specific (integrated) response: this could be interpreted as an example of antigen competition (*q.v.* Adler, 1964). By stimulating cell division,

pertussis, acting either as an antigen or an adjuvant, might 'push' antigen-sensitive cells into a non-reactive phase of the mitotic cycle. In general, the greatest adjuvant effect was seen when the sheep RBC and the pertussis were injected within a few minutes of each other.

Immune response expressed as the number of spleen cells which are making antibody at a particular time after immunization could be misleading if pertussis acted primarily outside the spleen and then stimulated the migration of antibody-producing cells into that organ. Consequently, the numbers of PFC in spleen, blood, peripheral lymph nodes and mesenteric lymph nodes, were measured at different times after the intraperitoneal injection of sheep RBC or sheep RBC plus pertussis. The results in Fig. 7 and Table 4 show that pertussis stimulated a net migration of PFC or their immediate precursors from the spleen to the lymph nodes, and not vice versa. It is clear that spleen responses will be under- not over-estimates of the total PFC in an animal.

It is not clear whether this increased migration is part of a generalized lymphocytosis. An intraperitoneal or intravenous injection of pertussis stimulates a large and immediate increase in the numbers of lymphocytes in the blood, an increase which is at the expense of the

TABLE 5. Direct ($-\log_2$) agglutination titres of pooled sera obtained from CBA mice

Serum	Titrated against pertussis	Titrated against sheep RBC
Primary anti-sheep RBC pool days 1–5	1	9
Primary anti-sheep RBC pool days 7–15	2	8
Primary anti-sheep RBC pool days 17–130	2	5
Secondary anti-sheep RBC pool days 4–14	2	10
Secondary anti-pertussis	14	0
Normal serum	1	1

lymphoid organs (Morse, 1965; Morse & Riester, 1967). This lymphocytosis is much reduced in thymectomized mice (Kalpaktsoglou, Yunis & Good, 1969), which suggests that pertussis acts by stimulating thymus-derived lymphocytes. However, PFC are almost certainly bone marrow-derived cells (Mitchell & Miller, 1968). In the experiments described in this paper, we have shown that pertussis leads to a considerable increase in circulating PFC (Fig. 7, Table 4), which leads us to conclude that pertussis stimulated lymphocytosis is probably not confined to thymus-derived cells.

It can be seen in Fig. 6 that pertussis injected together with 4×10^7 sheep RBC does not increase the rate at which γM- and γG_{2a}-PFC accumulate in the spleen: the response to 4×10^5 sheep RBC is too variable for any conclusion to be drawn with regard to the 'doubling time' of PFC or their immediate precursors. The higher number of PFC in pertussis-treated mice can be accounted for by a lengthening of the period during which the increase of PFC is taking place. The absence of an effect on the 'doubling time' suggests that pertussis might act by stimulating recruitment of antigen reactive cells (or antibody-producing cells) into the pool of proliferating cells, perhaps through increased trapping of circulating lymphocytes in lymphoid organs draining a site of injection (Taub, Krantz & Dresser, 1970; Dresser, Taub & Krantz, 1970).

In conclusion, we believe that adjuvants (and in a more restricted sense adjuvanticity—Dresser, 1961, 1968) act by stimulating cell proliferation. However, the situation is very complex, both with regard to the adjuvants themselves and with regard to the response of animals to injections of these substances. Consequently, generalizations made on the basis of experiments utilizing one particular system must be treated with caution. For instance, the antigen depot of Freund's adjuvant and the granuloma-stimulating activity of this adjuvant may both be of considerable importance in increasing antibody titres (Freund, 1953). Alum-precipitated bovine γ-globulin is highly immunogenic in mice. Nevertheless, either incorporation in Freund's complete adjuvant or admixture of pertussis organisms will lead to increased levels of antibody to this antigen preparation. However, if the two adjuvants are used together their effects are seen to be largely additive, suggesting that they may act in different ways, perhaps acting on two non-overlapping populations of antigen-sensitive cells (Dresser, unpublished experiments).

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