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On the mechanism of action of adjuvants

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Summary. Evidence is presented that one way which adjuvants exert their effect is by initiating an enduring increase in the localization of labelled cells in draining lymphoid tissues. Agents not generally considered to be adjuvants (carbon, latex, sheep erythrocytes) but capable of altering lymphocyte recirculation are shown to prevent the induction of tolerance by soluble bovine gamma globulin (BGG). These properties fulfil the criteria for adjuvanticity as defined by Dresser (1968) and link the expression of adjuvanticity to alterations in lymphocyte circulation.

INTRODUCTION

Substances known to have adjuvant properties can initiate marked changes in the pattern of lymphocyte recirculation (Frost & Lance, 1973). The subcutaneous injection of a variety of adjuvants initiates a highly significant increase in the localization of syngeneic lymphocytes in draining lymph nodes. This increased localization persists for as long as 45 days, i.e. as long as we have investigated these events (Frost & Lance, 1973). On the basis of these findings, we postulated that one mechanism by which adjuvants may have their effect is by an alteration of lymphocyte recirculation to produce a marked and enduring localization of cells in the draining lymph nodes. We further postulated that this enhanced localization of cells in draining lymph nodes would encourage optimal contact between antigen and antigen reactive cells, particularly when prolonged. Thus, antigen reactive cells which would otherwise not meet the antigen would now have antigen contact.

Dresser (1968) defined as an adjuvant any agent capable of aborting tolerance induction by aggregate-free bovine gamma globulin (BGG). We present evidence that agents which initiate changes in lymphocyte traffic, though they are not considered adjuvants, will abort tolerance induction by aggregate free BGG.

MATERIALS AND METHODS

Animals

All experiments utilized C3H female mice, aged between 6 and 10 weeks and obtained from the breeding unit at the Clinical Research Centre in London.

Agents tested for adjuvanticity

These included Freund's complete adjuvant (FCA)

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and latex particles (Difco Laboratories, Detrot, MI), sheep erythrocytes and *Corynebacterium parvum* (SRBC and *C. parvum*; Burroughs Wellcome Ltd., Beckenham, Kent), keyhold limpet haemocyanin (KLH; Calbiochem, Inc., CA), BGG (Armour Pharmaceuticals, England), silica (Dorentrup Quartz No. 12; provided by Dr A. C. Allison), and carbon particles (Pelican Ink C11/1431).

Preparation of tolerogenic BGG

A solution of BGG at 5 mg/ml in PBS was centrifuged at 40,000 *g* for 1 h. The top one-third of the clear supernatant was removed and the protein concentration estimated using the method of Lowry, Rosebrough, Farr & Randall (1951). The solution was diluted to a concentration of 400 μg/ml and 0·25 ml was injected intravenously into 6 week old C3H mice. The entire procedure was repeated 2 weeks later (Dresser, 1962).

Assessment of tolerance

One to 2 weeks after the last injection of tolerogenic BGG, the state of tolerance was assessed using the method previously described (Frost, 1974a; Dresser, 1965). Briefly, five tolerant and five control animals were injected with an emulsion of FCA containing 200 μg BGG. Seven days later, these, as well as unimmunized tolerant and control animals, were injected with 500 μg ^{125}I-labelled BGG intraperitoneally. The clearance of the labelled BGG was assessed by determining the count of radioactivity (c.p.m.) in 100 μl of tail vein blood every other day.

Preparation of antibody to BGG

Groups of C3H mice were injected with an emulsion of FCA containing 200 μg BGG intraperitoneally. Booster injections were given 2 and 5 weeks later (intraperitoneally to avoid anaphylaxis) and the animals were bled out 6 days after the last BGG booster injections. The sera were tested for their specificity by gel diffusion and radioimmunoassay (see below).

Radioactive labelling of lymphocytes

Single-cell suspensions of syngeneic lymph node cells were prepared as described previously (Frost & Lance, 1974). The cells were labelled *in vitro* with ^{51}Cr (supplied as sodium chromate Cr-51 by Radiochemical, Amersham, England) at 50 μCi/10^8 cells and washed twice prior to injection.

Demonstration of altered lymphoid cell traffic through stimulated lymph nodes

Labelled lymph node lymphocytes were injected intravenously into animals that had received antigen or adjuvant intravenously 1 h earlier or subcutaneously 24 h earlier. Twenty-four hours later, the animals were killed and the localization of labelled cells in the draining and control lymph nodes, spleen, and liver was determined by counting whole organs in a Packard gamma spectrometer.

Measurement of antibody to BGG

Titres of mouse anti-BGG antibody were determined by the radioimmunoassay described by Mitchison (1971). To 100 μl of 1·5 μg [^{125}I-]BGG (with at least 100 counts per second) was added 50 μl of a 1 : 10 dilution of the serum to be tested. Thirty microlitres of goat anti-mouse-globulin (Nordic) was then added and the mixture was allowed to sit at room temperature for 2 h. High controls (maximum precipitation) contained 0·5 ml of trichloroacetic acid in place of the anti-globulin, while low controls (essentially no precipitation) contained normal mouse serum in place of the test sera. After incubation, the tubes were spun and the supernatants removed. The precipitates were counted in a gamma spectrometer and the percent antigen binding calculated by computer, using the formula provided by Mitchison (1971).

Experimental design

The experiments described are based on the inability of soluble BGG to initiate changes in lymphocyte traffic through lymph nodes or spleen when injected subcutaneously or intravenously (Frost, 1974a; Frost & Lance, 1974). If, however, soluble BGG is injected into animals that had been previously passively or actively immunized to BGG, significant alterations in lymphoid traffic through draining lymphoid organs result (Frost, 1974a). We, therefore, postulated that, if changes in lymphoid traffic could be initiated prior to or at the same time as the injection of soluble BGG, then these alterations could optimize contact between lymphocytes and the soluble BGG and, thus, initiate a response to this tolerogen.

Particles such as carbon, latex, and silica, as well as antigens such as KLH and SRBC, were tested for their ability to block tolerance induction by aggregate-free (tolerogenic) BGG. Animals were injected with 100 μg of tolerogenic BGG (prepared as des-

cribed above), mixed with the following: 5×10^8 SRBC (IV); 650 μg KLH (IV); 4 mg carbon particles (IV); 0·2 cc latex particles (IV); or 3 mg silica (SC). Control animals received either intravenous BGG alone (negative control) or intraperitoneal FCA containing 100 μg BGG (positive control). Ten days later, all groups were challenged with tolerogenic BGG intravenously or subcutaneously and immunity was assessed by the following criteria: (1) the ability of the BGG challenge dose to induce increased localization of labelled cells in draining lymphoid organs; (2) the presence of anaphylaxis in response to BGG challenge; and (3) evidence for the presence of precipitating antibody 7 days after BGG boost.

RESULTS

Table 1 demonstrates that antigens (SRBC, KLH) and inert particles (carbon, latex, silica) all enhance the ability of tolerogenic BGG to increase the localization of labelled lymphoid cells in draining lymphoid organs. The increased localization of cells occurred whether the materials were injected intravenously as in the case of sheep cells, carbon, and KLH or subcutaneously as in the case of silica. The augmentation was equal to or greater than that observed with Freund's complete adjuvant. Control animals were injected with sequential doses of tolerogenic BGG and no augmentation in labelled cell localization was demonstrable when compared to control animals that had received sequential injections of saline. Since tolerogenic BGG has never been found to induce changes in lymphoid traffic in and of itself (Frost, 1974a; Frost & Lance, 1974), we attribute the alterations (Table 1) to an effect of the carrier agents. The direct effect of SRBC, carbon, etc., on lymphocyte recirculation wanes by 48–96 h. Thus, the demonstration of altered cell traffic on rechallenge with soluble BGG on day 11 cannot be attributed to the earlier injection. The fact that

Table 1. The induction of lymphoid cell traffic alterations by TBGG* in animals immunized with antigens of inert particles acting as adjuvants

Original immunization	Challenge dose of TBGG 10 days later	% Localization of labelled cells in the spleen	% Increase	% Localization of labelled cells in lymph nodes		% Increase
				Left	Right	
—	—	15·9 ± 0·8†	—	2·4 ± 0·08	2·5 ± 0·04	—
TBGG	100 μg IV	16·4 ± 0·6	—			
	100 μg SC‡			2·5 ± 0·1	2·7 ± 0·4	—
FCA + TBGG IP	15 μg IV**	22·0 ± 1·0	34			
	100 μg SC			4·1 ± 0·01	2·9 ± 0·3	64
SRBC + TBGG IV	100 μg IV	23·7 ± 1·7	45			
	100 μg SC			3·2 ± 0·1	2·5 ± 0·04	28
Carbon + TBGG IV	30 μg IV**	24·1 ± 2·4	47			
(same time)	100 μg SC			3·1 ± 0·07	2·1 ± 0·3	24
Carbon + TBGG IV	30 μg IV**	25·2 ± 1·6	54			
(1 h later)	100 μg SC			3·3 ± 0·6	2·3 ± 0·2	43
KLH + TBGG IV	100 μg IV	19·8 ± 1·3	21			
	100 μg SC			3·5 + 0·3	2·5 ± 0·03	40
Latex + TBGG IV	100 μg IV	23·9 ± 0·9	46	NT	NT	
Silica + TBGG SC	100 μg IV	26·2 ± 2·4	60	NT	NT	

* TBGG—Tolerogenic (soluble) BGG.

† Mean and standard deviation of three experiments (N = 12).

‡ All SC challenges given on left side.

** Challenge dose lowered to avoid anaphylaxis.

C3H mice were injected on Day 0 with 100 μg of tolerogenic BGG alone or as an emulsion with 0·2 ml FCA or in association with 5×10^8 SRBC, 650 μg KLH, 4 mg carbon, 0·2 cc latex particles, or 3 mg of silica. One group received 4 mg of carbon intravenously 1 h prior to the TBGG. All groups were challenged with TBGG 10 days after the initial immunization followed by ^{51}Cr-labelled syngeneic lymphocytes. Animals were killed 24 h later and the organ distribution of labelled cells assessed in a gamma spectrometer.

carbon, injected an hour before BGG, produced the same effect, is evidence that the effect is not due to a physical association between BGG and the material conferring adjuvanticity.

Table 2 demonstrates that animals so injected are immunized. Immunity was measured in two ways. The first, the induction of anaphylaxis, was performed in a double blind manner. The observer was not aware of the immune status of the animals tested. The induction of anaphylaxis was dose dependent, with 10 to 20 μgm of tolerogenic BGG being effective in the FCA and silica groups, while other groups required 100 μgm before symptoms could be demonstrated. Within 10 to 30 min of challenge, sensitized animals became listless and apathetic, becoming immobile a short time afterwards. The fur became ruffled and cyanosis of the limbs and impaired respiration developed soon afterwards. All animals then developed diarrhoea and most succumbed within 1 to 2 h after antigen challenge. On autopsy, all groups demonstrated severe hyperaemia, oedema, and distension of the small bowel. In several cases, perforation of the small bowel had occurred. Animals that did not succumb within 2 h but appeared grossly ill were killed and autopsied. The results at autopsy were similar but not as severe as those observed in the animals that had succumbed within 2 h. Death within 2 h was graded as a 4+ reaction. The 3+ and 2+ reactions were based on subjective criteria—the degree of hyperaemia, the oedema of the bowel, and the illness of the animal. Anaphylaxis occurred in all animals that had been sensitized previously with BGG in association with either antigen, adjuvant or inert particles.

Table 2. The demonstration of anaphylaxis and antibody production after immunization with tolerogenic BGG given in conjunction with unrelated antigens or inert particles

Immunization	Anaphylaxis	Antigen-binding capacity
TBGG Alone	0	ND*
FCA + TBGG	4+	> 1·8 μg/ml
SRBC + TBGG	2+	0·22 μg/ml
Carbon + TBGG (same time)	2+	0·71 μg/ml
Carbon + TBGG (1 h later)	2+	0·74 μg/ml
KLH + TBGG	2+	0·21 μg/ml
Latex + TBGG	2+	0·16 μg/ml
Silica + TBGG—SC	3+	> 1·8 μg/ml

* Non detectable.

Groups of mice were injected with TBGG alone or in conjunction with KLH, SRBC, carbon, latex, or silica. Ten days later, all animals were challenged with TBGG at the dose indicated. Anaphylaxis was assessed within 2 h (one half the animals) and antigen binding capacity was determined 7 days after challenge.

Further evidence for the enhanced immunogenicity of BGG injected together with adjuvant-like substances was demonstrated by the assessment of the antigen binding capacity in the serum of animals so immunized. These data are also presented in Table 2. Animals immunized with tolerogenic BGG in conjunction with the agents utilized were kept for 20 days and rechallenged with 100 μgm of tolerogenic BGG intravenously. All groups demonstrated detectable levels of antibody to BGG. Control animals received only tolerogenic BGG on the initial immunization and challenge. These groups never demonstrated detectable antibody.

DISCUSSION

Adjuvants induce more marked and prolonged alterations in the circulation of lymphocytes through draining lymphoid tissues than do antigens (Frost & Lance, 1973). We have now demonstrated that antigens and inert particles can also express adjuvant properties by altering lymphocyte traffic. Carbon, latex, silica, SRBC and KLH, injected into mice with tolerogenic BGG, increase the localization of cells in draining lymphoid tissues and enhance the immune response to this antigen. This procedure prevents induction of tolerance by BGG which by the definition of Dresser (1968) demonstrates an adjuvant action of these agents.

We have previously shown that the macrophage is important for the initiation of lymphocyte traffic changes induced by antigens, adjuvants, or particulate material. Evidence supporting this role for the macrophage (and not lymphocytes) is based on the fact that the change induced by antigens is resistant to X-irradiation, cyclophosphamide, antilymphocyte serum, and hydrocortisone (Frost & Lance, 1974). In addition, X-irradiated nude mice, when challenged with antigen, show changes in lymphocyte recirculation identical to those seen in normal animals (Frost & Lance, 1974).

Long established means of inducing adjuvant

effects have involved the precipitation of antigen with aluminium hydroxide or aluminium phosphate (Glenny, Pope, Waddington & Wallace, 1926; White, Coons & Connolly, 1955a). Diphtheria toxoid prepared in this way acts as a far better immunogen than the native antigen (Edsall, 1946). Materials such as bentonite (Claman, 1963; Gallily & Garvey, 1968) and latex (Litwin & Singer, 1965) which are also used to present antigen in a particulate form, increase the immune response, probably by enhancing phagocytosis (Torrigiani & Roitt, 1965; Edsall, 1965). Dresser (1961, 1965) has clearly shown that while soluble BGG is tolerogenic, aggregated BGG is immunogenic. These differences in immunogenicity coincide with the effects of soluble and particulate BGG on lymphocyte recirculation. Soluble BGG does not initiate recirculatory changes unless injected into animals that have been passively or actively immunized against BGG (Frost, 1974a). Heat aggregated or alum precipitated BGG cause changes of cell traffic in both normal and tolerant animals comparable to that seen with other particulate antigens (Frost, 1974a, b).

These reports, coupled with other studies, link adjuvant action to macrophage function. White *et al.* (1955b) reported marked reticuloendothelial cell proliferation at the site of injection of wax D (derived from mycobacteria) as well as in the draining nodes. Similar stimulatory effects on reticuloendothelial cells and phagocytosis have been described after the injection of endotoxin (Boehme & Du Bos, 1958; Rowley, 1960), FCA (Laufer, Tal & Behar, 1969; Rupp, Moore & Schoenberg, 1960), *C. parvum* (Howard, Scott & Christie, 1973), and oligonucleotides (Braun & Firshein, 1967; Freedman & Braun, 1965). Antigen taken up by macrophages and adoptively transferred to syngeneic hosts, results in a better immune response than antigen alone (Unanue & Askonas, 1968; Spitznagel & Allison, 1970). The adoptive transfer of adjuvant (beryllium salts or *Bordetella pertussis*) in macrophages with or without antigen in the same cell, leads to a much higher antibody response than transfer of macrophages containing antigen alone (Unanue, Askonas & Allison, 1969). Similar findings have been reported with polynucleotides (Johnson & Johnson, 1971).

The relationship between macrophages and adjuvants is thus well established, as is the link between macrophage function and altered lymphocyte traffic. It seems a reasonable hypothesis, then, that changes in lymphocyte recirculation are related to the mechanisms by which macrophages exhibit their adjuvant effects.

In the Dresser terminology (1968), the difference between a good and a poor immunogen (a particulate or soluble antigen) is related to the intrinsic adjuvanticity of the antigen. Thus, an immunogen is a good immunogen because by its intrinsic adjuvanticity it enhances the immune response to itself. Since all good immunogens tested enhance the localization of labelled cells in draining lymphoid tissue (Frost, 1974a, b; Frost & Lance, 1974), this property could be the basis for expression of intrinsic adjuvanticity.

Similarly, 5×10^6 SRBC induce a less than optimal immune response in mice, but at this dose, sheep erythrocytes do not alter lymphocyte recirculation. Five $\times 10^8$ SRBC alter cell traffic and induce an optimal immune response (Frost, 1974b). Thus, the dose dependence of any immunogen may be related to its ability to express intrinsic adjuvanticity by altering lymphocyte recirculation.

A variety of substances express extrinsic adjuvanticity, including micro-organisms (mycobacteria, *B. pertussis*, *C. parvum*) and non-immunogenic materials (vitamin A, beryllium salts, and oligonucleotides). Because of this diversity in materials with adjuvant properties, a unified concept of how adjuvants work has been difficult to formulate. Our observation that all adjuvants tested initiate changes in lymphocyte recirculation which result in the abrogation of tolerance and enhancement of immunity may provide a basis for explaining some aspects of the expression of adjuvanticity.

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