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THE MECHANISM OF TOLERANCE IN CONTACT HYPERSENSITIVITY TO DINITROCHLOROBENZENE IN GUINEA-PIGS

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SUMMARY

Guinea-pigs made tolerant to dinitrochlorobenzene (by prior intracardiac injection of dinitrobenzene sulphonate) failed to give positive skin reactions after contact sensitization, but nevertheless had peritoneal cells which reacted with the hapten in macrophage migration inhibition (MMI) tests. This reactivity was blocked *in vitro* by the addition of serum from tolerant animals but not by serum from hypersensitive animals.

Cells from hypersensitive guinea-pigs were anomalous, in that their reaction with hapten in MMI was not blocked by tolerant serum. Hypersensitive serum, though not active by itself in MMI, was able to prevent blocking by tolerant serum when the two sera were mixed. This was interpreted as an 'unblocking' phenomenon and suggested that hypersensitive cells were insusceptible to blocking because they themselves produced an unblocking substance (antibody?), although preliminary efforts to demonstrate this directly were not successful. Hypersensitive serum had an analogous activity *in vivo*, since when passively transferred to otherwise tolerant animals it enabled them to produce typical skin reactions; that is, it broke tolerance.

Tolerance or non-reactivity *in vivo* in the situation investigated thus appears to be an enhancement-like process, characterized by the presence of reactive lymphoid cells and a blocking factor (antigen–antibody complex?) detectable in the serum of the tolerant animals.

INTRODUCTION

Many simple chemicals, when applied to the skin, cause the development of a generalized hypersensitive state so that a later application to a different skin area results in a typical allergic reaction of the delayed type (Chase, 1967). This is contact hypersensitivity or contact dermatitis. The chemicals appear to act as haptens by coupling with skin proteins

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to induce the allergic response (Eisen & Tabachnick, 1958); skin reactivity is then a cell-mediated phenomenon, transferable to normal recipients by lymphoid cells but not by serum (Chase, 1967). Of particular interest is the observation of Sulzberger (1929) and Chase (1946; Chase & Battisto, 1959) that doses of certain haptens administered by a different route prior to epicutaneous application appeared to render animals immunologically tolerant. Much detailed work has been done with picryl chloride or related compounds fed to guinea-pigs (Chase, 1946, 1967; Chase & Battisto, 1959; Frey *et al.*, 1972) or injected into mice (Asherson & Ptak, 1970), and with dinitrobenzene derivatives injected into guinea-pigs (Frey, De Weck & Geleick, 1964a, b). Application of the same or related chemicals to the skin, after an interval of several days or more, then fails to induce the expected hypersensitive state as shown by subsequent skin-testing. The animals are made refractory by prior antigen exposure.

Acquired tolerance of the type described is usually conceived as a specific lack of immunological responsiveness, the first exposure to a large antigen dose causing destruction of potentially responsive cells (Chase & Battisto, 1959; Frey *et al.*, 1972). Some recent studies of other types of immunological non-reactivity have disclosed positive cellular activity *in vitro*, accompanied by serum factors which interfere with its manifestation (Hellström & Hellström, 1970, 1973; Voisin, 1971; Halliday, 1972a). We have now used the MMI technique to show that guinea-pigs made hypersensitive and tolerant towards 2,4-dinitrochlorobenzene (DNCB) have cells which are reactive with this hapten. In addition, the sera of these animals were examined for their ability to prevent or 'block' the reaction between cells and hapten. MMI is a well-recognized means of detecting cell-mediated immunity (CMI) *in vitro* (David *et al.*, 1964), and has been used to demonstrate cellular reactivity and serum blocking factors in relation to tumour antigens (Halliday, 1972b). Finally, simple *in vivo* experiments demonstrated the rapid breakdown of tolerance and confirmed the prior existence of reactive cells.

MATERIALS AND METHODS

Guinea-pigs (weight about 500 g) of a randomly bred strain were prepared as follows. Animals to be made tolerant were given 250 mg of 2,4-dinitrobenzene sulphonic acid (DNBS) sodium salt (Eastman Kodak, Rochester, New York) intracardially in 2 ml of saline, 7 days prior to the sensitizing dose of 1 mg of DNCB (British Drug Houses, Poole, England), which was applied to the shaven skin of the neck in 0·002 ml of acetone solution. Animals to be hypersensitized received the same skin application of DNCB but no DNBS.

The skin-test doses of DNCB (22·5, 12·5 and 7·5 μg in 0·025 ml of acetone) were applied to the shaven flank 7–14 days after sensitizing and reactions were read on the following day. The intensity of these reactions was recorded by the method of Frey *et al.* (1964b). In this procedure, isolated red spots on the test area corresponded to a reading of 0·5; diffuse redness, 1; marked redness and slight swelling, 2; deep redness and considerable swelling, 3. The sum of the readings obtained with the three different test concentrations was used as a measure of degree of hypersensitivity. Hypersensitized animals always exhibited skin reactions with a score of 2·5–3·5, whilst the tolerant group was uniformly non-reactive. Normal control guinea-pigs were skin-tested and they also were non-reactive. Mineral oil (liquid paraffin, 30 ml) was injected intraperitoneally 4 days before skin-testing, when exudates were required to provide cells for the MMI technique.

On the day after skin-testing, animals from all groups (tolerant, hypersensitized and normal) were bled to provide serum, then peritoneal exudate cells (PEC) were harvested from the killed animals by washing out their abdominal cavities with Medium 199 (Commonwealth Serum Laboratories, Melbourne). Standard procedures (Bloom & Bennett, 1971; Halliday, 1972b) were employed in setting up MMI reactions, PEC from each group being incubated in capillary tubes in Medium 199, with 35 mm × 10 mm tissue culture dishes (Falcon Plastics, Oxnard, California) as migration chambers. Guinea-pig serum, from the same or similar animals, was incorporated into the culture medium at 15% concentration; no other serum was used. Antigen was also added to the medium where required, as the specific water-soluble hapten DNBS (0·1 mg/ml). Areas of cell migration (after 18 hr incubation at 37°C in a 5% CO_2 = air atmosphere) were traced with the aid of a camera lucida and measured by planimetry.

Cultures of PEC were made by suspending the washed cells in Medium 199 at a density of 4×10^6/ml, and incubating in 5 ml quantities in tissue culture dishes, for 18 hr at 37°C (Halliday, 1972b). The fluids were then centrifuged at $200 \times g$ for 5 min to remove cells and debris.

For convenience, PEC and serum from tolerant animals will be called 'tolerant PEC' and 'tolerant serum', and similarly for hypersensitive animals. The term 'tolerant animal' is used in an operational sense, meaning an animal which cannot be hypersensitized to give positive skin reactions, by the standard techniques successful with normal animals.

RESULTS

Reactivity of cells and blocking by serum

The results of two separate experiments are shown in Table 1. As might have been expected, PEC from hypersensitive guinea-pigs migrated well with normal serum and this migration was inhibited by the addition of DNBS (Table 1a). Thus MMI can be used as an *in vitro* index of contact hypersensitivity to DNCB. DNBS, in the concentration used, had no effect on the migration of normal cells. A less predictable finding was that so-called tolerant PEC, from animals which lacked skin reactivity *in vivo*, behaved in a manner similar to hypersensitive PEC; that is, they exhibited MMI in the presence of the hapten and normal serum.

The findings of reactivity in tolerant PEC prompted an investigation of the properties of serum from the same animals. As shown in Table 1b, tolerant serum behaved quite differently from normal serum; in contrast to the latter, it prevented the reaction between hapten and tolerant cells and thus partially or completely blocked MMI in these mixtures. Under the conditions used, hypersensitive PEC were not blocked by tolerant serum. Hypersensitive serum appeared to be similar to normal serum and did not block MMI (Table 1c).

Further in vitro investigations with hypersensitive PEC and serum

The resistance of hypersensitive PEC to blocking by tolerant serum was for a long time a puzzling enigma. Eventually, a consideration of possibly analogous phenomena in tumour immunology led to the suggestion that an 'unblocking' mechanism might be operative, and that hypersensitive PEC and serum might contain a material which interfered with blocking. Accordingly, hypersensitive PEC were cultured as described above and the culture fluid mixed with a system exhibiting blocking (tolerant PEC + antigen + tolerant serum); no diminution of blocking was found in several trials. However, attempts to demonstrate

unblocking by hypersensitive serum were more rewarding. As shown in Table 2, hypersensitive serum (although having no direct effect on MMI as seen previously) cancelled the blocking activity of tolerant serum in a 1:1 mixture. This was not merely a dilution effect; in several other experiments, an equal amount of normal serum did not reduce blocking by tolerant serum.

TABLE 1. Macrophage migration with PEC from hypersensitive, tolerant and normal guinea-pigs, with and without antigen, in the presence of serum from different donor animals

PEC donors	Antigen (DBNS)	Migration areas* Experiment 1	Experiment 2	Result
(a) In medium containing normal serum				
Hypersensitive	–	45, 55, 51 (50)	110, 90, 118 (106)	Inhibition
	+	38, 20, 35 (31)	65, 62, 56 (61)	
Tolerant	–	62, 62, 66 (63)	145, 141, 130 (139)	Inhibition
	+	38, 37, 35 (37)	40, 34, 40 (38)	
Normal	–	50, 60, (55)	111, 93, 119 (108)	No inhibition
	+	51, 50, 49 (50)	118, 99, 108 (108)	
(b) In medium containing tolerant serum				
Hypersensitive	–	47, 52, 51 (50)	160, 162, 180 (167)	Inhibition
	+	14, 13, 18 (15)	94, 90, 92 (92)	
Tolerant	–	40, 56, 40 (45)	142, 90, 128 (120)	No inhibition
	+	82, 68, 85 (78)	90, 125, 105 (107)	(blocking)
Normal	–	62, 70, 46 (59)	115, 100, 117 (111)	No inhibition
	+	50, 49, 55 (51)	167, 90, 128 (128)	
(c) In medium containing hypersentitive serum				
Hypersensitive	–	82, 74, 87 (81)	120, 126, 112 (119)	Inhibition
	+	29, 28, 20 (26)	75, 61, 60 (65)	
Tolerant	–	75, 60 (67)	140, 123 (131)	Inhibition
	+	12, 10, 13 (12)	25, 31, 45 (34)	
Normal	–	85, 107, 71 (88)	140, 124, 105 (133)	No inhibition
	+	77, 70 (74)	147, 140, 129 (139)	

* Areas are expressed in arbitrary units, constant within each experiment; individual measurements of replicates are shown, with means in parentheses.

Hypersensitive serum and unblocking in vivo

Serum from hypersensitive guinea-pigs, shown to have unblocking activity in MMI *in vitro* (Table 2), was injected in 10-ml quantities into tolerant guinea-pigs by the intraperitoneal route. Shortly afterward (on the same day) the recipients were skin-tested in the usual way and were found to have converted to hypersensitivity. The intensities of their reactions before serum administration, 24 hr after and 2 weeks after, are recorded in Table 3. Only guinea-pig number 2 failed to give a marked reaction after receiving the serum; it scored 0·5 only with the highest concentration of DNCB and had reverted to full tolerance 2 weeks

later. The other three animals retained skin-reactivity for a considerable time, at a diminishing level. Guinea-pig number 3 gave reactions of intermediate degree at 24 hr.

The positive reactions at 24 hr (Table 3b) were examined histologically after skin biopsy, sectioning, and staining with Haematoxylin and Eosin. Guinea-pigs numbers 1, 3 and 4 showed marked lymphocytic infiltration of the dermis, number 4 having the most extensive cellular reaction. Number 2, which gave only slight visible reactions, was histologically normal.

TABLE 2. Effect of hypersensitive serum on blocking of MMI

PEC donor	Serum	Antigen (DBNS)	Migration areas	Result
Tolerant	Normal	–	269, 267, 205 (247)	Migration
		+	167, 194, 186 (182)	inhibited 26%
Tolerant	Tolerant	–	189, 226, 192 (202)	Migration
		+	224, 221, 229 (231)	not inhibited (blocked)
Tolerant	Tolerant and hypersensitive	–	229, 220, 228 (226)	Migration
			167, 177, 175 (173)	inhibited 23% (unblocked)

TABLE 3. Effect of hypersensitive serum administration on tolerant recipients' skin reactivity *in vivo*

Time of testing	Skin reactivity to DNCB guinea-pig number			
	1	2	3	4
(a) Before administration of serum	0	0	0	0
(b) 24 hr after administration	3·5	0·5	2·0	3·5
(c) 2 weeks after administration	3·0	0	1·5	1·5

Normal guinea-pigs given the same dose of the same hypersensitive serum, then skin-tested, exhibited entirely negative reactions.

The newly reactive recipient guinea-pigs were bled 24 hr after serum administration (just after their skin reactions were read) and the sera were tested for blocking activity *in vitro*. The results are shown in Table 4. There was a good correlation between migration inhibition and skin reactivity (Table 3b): the least reactive animal (number 2) had serum which was still partly blocking and the most reactive (numbers 1 and 4) had completely unblocked serum; number 3 occupied an intermediate position in both tests. Although these particular animals had not been bled previously, tolerant serum has consistently proved to be blocking (Tables 1 and 2).

CMI in tolerant animals was thus revealed *in vivo* by the same serum which was unblocking *in vitro*.

TABLE 4. Reactivity of recipients' sera in MMI tests, 24 hr after administration of hypersensitive serum

PEC donor	Serum from guinea-pig number	Antigen (DBNS)	Migration areas	Result
Tolerant	1	–	209, 236, 226, (224)	Migration
		+	149, 176, 168 (164)	inhibited 27%
				(unblocked)
Tolerant	2	–	188, 200, 239 (209)	Migration
		+	180, 193, 195 (189)	inhibited 10%
				(partly blocked)
Tolerant	3	–	192, 188, 200 (193)	Migration
		+	154, 158, 155 (156)	inhibited 19%
				(unblocked)
Tolerant	4	–	200, 226, 242 (223)	Migration
		+	163, 147, 170 (160)	(unblocked)

The same PEC were used in the experiment of Table 2, where they showed inhibition by antigen in the presence of normal serum and blocking by tolerant serum.

DISCUSSION

On the evidence presented, guinea-pigs made tolerant to DNCB appear to have reactive or sensitized cells, presumably lymphocytes. These apparently react with the hapten and liberate lymphokines which inhibit macrophage migration *in vitro*. Tolerant animals are thus characterized, not by the absence of cell-mediated responsiveness as has usually been inferred from observations *in vivo*, but by the possession of specific circulating factors demonstrable in their serum by the blocking of reactivity *in vitro*. It is anticipated that similar mechanisms will be found to operate in tolerance induced towards other contact sensitizers.

In our hands, the demonstration of skin non-reactivity in intracardially injected animals and of reactive PEC together with blocking serum, has been completely reproducible; all of these phenomena were constantly observed with guinea-pigs of different origins and with experiments conducted in two different laboratories over many months.

Although blocking was demonstrable *in vitro* only with tolerant PEC, it is considered that hypersensitive PEC were potentially 'blockable' but may have been producing an unblocking factor. This has not yet been detected directly, as have PEC-produced blocking factors in other circumstances (Halliday, 1972b). It is possible that concentrating the PEC culture fluids may have led to positive results. On the other hand, the fact that hypersensitive animals produced an unblocking factor *in vivo* was clearly shown by the effect of hypersensitive serum on a blocked PEC–antigen system *in vitro*.

The probable nature of the blocking and unblocking factors operative in DNCB contact hypersensitivity is suggested by analogous findings in tumour immunity. Here it has been proposed that blocking is a function of antigen–antibody complexes and unblocking is caused by free antibody (Sjögren *et al.*, 1971; Baldwin, Price & Robins, 1972; Hellström & Hellström, 1973). This is consistent with the stimulation of both reactive lymphocytes and

antibody by DNCB contact sensitization, whereas the large prior tolerizing dose of DNBS seems to elicit antibody and combine with it to give circulating blocking factor, as long as the supply of hapten lasts. Hypersensitive serum is presumably unblocking because it contains free antibody, which may unbalance the proportions of hapten and antibody required for blocking action. Further experiments are planned to characterize the blocking complex hypothetically formed with DNBS and to locate its site of action.

There is no dearth of experimental data in the literature concerned with transfer of cells or serum in research into tolerance. In the field of transfer experiments used for investigating the present type of phenomenon, it is difficult to devise a procedure which has not been used before, although the interpretations may be questioned. Using guinea-pigs made hypersensitive and tolerant to picryl chloride (by skin painting and oral administration, respectively), Chase and his colleagues performed many transfer experiments, including the following.

(a) Hypersensitive cells (blood leucocytes, lymph node or spleen cells) transferred to normal recipients led to hypersensitivity in the latter, especially if reinforced by the antigenic stimulus of skin testing (Chase & Battisto, 1959; Chase, 1967). Similar cells administered to tolerant animals also led to hypersensitivity in the recipients, but this was more transient (Chase & Battisto, 1959).

(b) Tolerant cells transferred to normal guinea-pigs produced neither hypersensitivity nor tolerance in the recipients (Battisto & Chase, 1963). It was emphasized that the guinea-pigs were not isogeneic, so were probably not histocompatible and the transferred cells would not have survived long. Analogous experiments done with inbred mice (Asherson, Zembala & Barnes, 1971) gave the opposite result, namely that tolerant cells imposed their properties on normal animals.

(c) Hypersensitive serum administered to normal animals did not transfer delayed hypersensitivity (Chase & Battisto, 1959). When given to tolerant guinea-pigs it was eliminated at the same rate as in normal recipients (Chase & Battisto, 1959) but the tolerant animals seem not to have been skin-tested after serum transfer.

(d) Tolerant serum given to hypersensitive animals did not interfere with their skin reactivity and thus appeared to contain no blocking antibody (Chase & Battisto, 1959).

Asherson *et al.* (1971) found that lymph node cells of normal mice restored immune competence to irradiated tolerant animals; tolerant cells were unable to do this and furthermore they interfered with the restorative properties of normal cells and of sensitized cells. These results were interpreted as indicating antibody-mediated depression (blocking) of hypersensitivity in tolerant animals.

Suitable *in vitro* tests facilitate the analysis of *in vivo* phenomena by separating different facets of immune reactivity. Thus when PEC of DNCB-tolerant guinea-pigs were washed and tested in MMI, they were unequivocally reactive with the hapten. The addition of tolerant serum led to blocking of the reaction and this seems to be analogous to the apparently unresponsive state *in vivo*. Hypersensitive animals produced a substance which endowed their serum with unblocking powers, so that MMI was regained by blocked PEC *in vitro* (Table 2) and skin-reactivity was restored to tolerant animals *in vivo* (Table 3). When tolerant serum was given to hypersensitive animals (see (d) above) it must have encountered this substance and was therefore ineffective in abrogating skin reactivity. The fact that hypersensitive cells were not blockable in MMI (Table 1b) is consistent with their previously observed ability to transfer hypersensitivity to tolerant animals (see (a) above) and might be explained by their elaboration of unblocking antibody. It is thus easy to understand

(without invoking theories of clonal deletion) the ability of normal and especially hypersensitized cells to break tolerance, in this and perhaps other situations, and the inability of tolerant serum to diminish hypersensitivity.

When hypersensitive serum was injected into tolerant guinea-pigs it promptly broke their tolerance and skin tests became positive. The same serum did not induce skin-reactivity in normal recipients, thus eliminating any possible confusion with immediate-type (antibody-mediated) hypersensitivity, Arthus reactions, or 'arming' of normal cells by antibody.

Although tolerant serum seemed to possess blocking factors, supposedly containing antibody, many studies (Chase & Battisto, 1959; Chase, 1967; Frey *et al.*, 1972) have demonstrated depression of serum antibody levels in tolerance. Antibody is conventionally measured by passive cutaneous anaphylaxis or passive haemagglutination. These observations are compatible if it is assumed that antibody in the form of blocking factors from tolerant animals is serologically inert since it is already complexed with antigen. The latter must also be in a partially inactive form, since it does not activate sensitized lymphocytes.

Blocking of the *in vitro* manifestations of CMI, by factors detectable in serum, has been shown to be functional in several other situations; in tumours, in transplants and in foetuses (Hellström & Hellström, 1970; Halliday, 1972a). The phenomena in tumour immunity are exactly analogous to those presently described. Thus CMI and serum blocking factors are found by *in vitro* methods in 'tolerant' subjects unable to reject their tumours (Hellström & Hellström, 1973); 'immune' individuals, after removal or regression of tumours or treatment with various vaccines (and, incidentally, most reactive in skin tests for delayed hypersensitivity to tumour antigens), produce serum which is unblocking *in vitro*; and this serum restores reactivity *in vivo* as shown by its positive effect in inhibiting tumour growth (Bansal & Sjögren, 1972). Although the last type of observation (suppression of tumour growth by immune serum) could be a function of a complement-dependent cytotoxic antibody, rather than one which unblocks CMI, this interpretation is clearly not permissible for DNCB-tolerance where antigenic cells are not involved. Similarly, the ability of hyperimmune serum to abrogate tolerance of certain skin allografts (usually explained as the elimination of cellular chimaerism; see Billingham & Silvers, 1971) could be a consequence of unblocking, especially in cases where the results appear rapidly. Once again, the findings reported in this paper cannot be explained by the destruction of antigenic cells or the removal of a tolerance-maintaining antigen in some other form.

A possible complication of the present observations and interpretations is that CMI is thought to have its specificity directed largely towards the carrier portion of a complete antigenic molecule, rather than towards the hapten portion (David, Lawrence & Thomas, 1964). Here we have given our attention entirely to hapten-specific CMI. In justification, it should be emphasized that all tests were made without introducing xenogeneic proteins, so that presumably the autochthonous carrier proteins bearing the dinitrophenyl hapten were similar throughout and could be ignored. Borel & David (1972) found that tolerance to hapten-protein conjugates was associated with lack of MMI towards the same conjugates, but in this case the foreign protein carrier must have been largely responsible.

In contrast to the conventional interpretation of *in vivo* experiments, it has been shown that tolerance in regard to contact sensitizing haptens is a positive phenomenon involving both reactive cells and blocking factors. These were readily detected by MMI experiments *in vitro*, and furthermore the antagonistic or unblocking properties of serum from hypersensitive animals indicated a distinction between blocking factors and free antibody. The

ability of hypersensitive serum to break tolerance *in vivo* (simulating the transfer of CMI by serum!) confirmed that reactive cells were already present in tolerant animals and that blocking factors were functional in maintaining non-reactivity.

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