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Non-Specific Inhibition of the Immediate and Delayed Types of Hypersensitivity during Immune Paralysis of Adult Guinea-Pigs

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Summary. Immunological paralysis induced in adult guinea-pigs with various protein overloading treatments is specific only at the beginning and soon after the end of the paralysis-inducing treatment. Reported experiments show that during a period which starts between the 8th and 10th days of the paralysing treatment and finishes when this treatment is discontinued, there is established an unresponsiveness towards antigens unrelated to the paralysing one. This non-specific unresponsiveness is manifested by the inhibition of reactions of both immediate and delayed types of hypersensitivity, at least to certain antigens.

INTRODUCTION

Immunological paralysis and tolerance are thought to be as specific as immunity. Classical studies by Billingham, Brent and Medawar (1953) established that mice rendered tolerant by embryonic injection of cells will accept skin-grafts only originating from the donor strain. Dixon and Maurer (1955a) injected in adult rabbits large quantities of human plasma. These rabbits became unresponsive towards the major constituents of the plasma (albumin, γ-globulin) but they produced antibodies against the minor constituents (α- and β-globulins). Felton, Kauffmann, Prescott and Ottinger (1955) studied cross-reactivity in mice paralysed with types I, II or III pneumococcal polysaccharides and found that paralysed mice could be immunized when injected with the other two pneumococcal polysaccharides. However there was, in mice paralysed with type I polysaccharide, some inhibition of later antibody formation against type II polysaccharide. Weigle (1961) found that albumins cross-reacting by more than 30 per cent with bovine serum albumin did not sensitize rabbits rendered tolerant to BSA by one neonatal injection, while those cross-reacting to a less extent with BSA did sensitize them. Similarly, Cinader and Dubert (1955) showed that two of six rabbits rendered tolerant to human serum albumin (HSA) reacted by an immune response to injections of benzene-*p*-sulfonic acid–azo–HSA which extensively cross-reacts with rabbit anti-HSA.

Concerning cross-tolerance towards unrelated antigens, contradictory data had been reported from studies on immunological paralysis: Hanan and Oyama (1954) reported that production of anti-ovalbumin antibodies in rabbits rendered tolerant to bovine serum albumin (BSA) by neonatal injections was significantly reduced; Hirata and Schechtman (1960) observed a similar falling off of the antibody response to BSA in chickens tolerant to human γ-globulin (HGG) and Timourian and Schechtman (1962) found a repression

of anti-HGG and anti-BSA production in rabbits tolerant to bovine γ-globulin (BGG). Nevertheless Smith and Bridges (1958) did not observe any significant depression of anti-ovalbumin production in rabbits tolerant to BSA, nor did Hirata, Garvey and Campbell (1960) find that injection of [^{35}S] BSA in chickens at hatching affected the subsequent antibody response to HGG.

The present paper reports the results of our studies on the specificity of the immunological paralysis induced in adult guinea-pigs by injection of high doses of heterologous proteins. Preliminary studies (Liacopoulos, 1961; Liacopoulos, Halpern and Perramant, 1962a; Liacopoulos, Neveu, Biozzi and Halpern, 1962) have shown that under certain conditions, non-specific unresponsiveness may be observed as a consequence of such a treatment. Complementary data and information concerning this matter will be reported here.

METHODS AND MATERIALS

Animals

The experiments were performed on adult male or female guinea-pigs, weighing 400–500 g. In studies dealing with the delayed type of hypersensitivity, strain K albino guinea-pigs were used.

Antigens

Immunological paralysis was induced by injections of the following antigens: bovine serum albumin (BSA) (Armour or Pentex), human serum albumin (HSA) (Centre National de la Transfusion Sanguine), bovine γ-globulin (BGG) (Armour), human γ-globulin (HGG) (Mérieux). Sensitization of the animals was performed with the following antigens: hen ovalbumin (EA) three times recrystallized, prepared in the laboratory according the method of Kekwick and Cannan (1936); rabbit γ-globulin (RGG) prepared by precipitation with ammonium sulphate at 33 per cent of saturation of rabbit serum; picrylated guinea-pig serum albumin (Pic.Gp.SA) prepared according to the method of Benacerraf and Gell (1959).

Induction of Immunological Paralysis

Guinea-pigs were injected daily with the above proteins according to the following scheme:

BSA: 100–800 mg./day by intravenous (i.v.) (1/3) and intraperitoneal (i.p.) (2/3) routes.

HSA: 600 mg./day, intravenously (i.v.) (1/3) and intraperitoneally (i.p.) (2/3) and 140 mg., every other day, locally in the four foot-pads.

BGG: 400 mg./day i.v. (1/3) and i.p. (2/3) and 65 mg., every other day, locally in the four foot-pads.

HGG: 300 mg./day i.v. (1/3) and i.p. (2/3), and 50 or 60 mg., every other day, in the four foot-pads or the two hind foot-pads.

Treatment with these proteins was started on the day of sensitization or several days before it, and was stopped 4–7 days after sensitization.

Sensitization

For the induction of the immediate type of hypersensitivity (anaphylaxis), sensitization

was performed by an intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) injection of 10 mg. of alum precipitated EA or RGG. In one experiment, animals injected with alum precipitated RGG were reinjected, 3 months later, intravenously with RGG, labelled with ^{131}I according to the method of Francis, Mulligan and Wormall (1951) and samples of blood were taken daily in order to follow the rate of disappearance of the labelled antigen from the circulation.

The delayed type hypersensitivity was induced by injection of 12·5 μg. of Pic.Gp.SA incorporated in 0·1 ml. of complete Freund's adjuvant (Difco), intradermally (i.d.) in each of the four foot-pads or in the hind foot-pads.

Evaluation of the State of Hypersensitivity

Data concerning the immediate type of hypersensitivity (anaphylaxis) was obtained by three of the most sensitive methods:

(a) Schultz-Dale reaction using ileal strips from treated and control animals (Bier, Siqueira and Beraldo, 1961).

(b) Passive cutaneous anaphylaxis (Ovary, 1958).

(c) Passive haemagglutination, using rabbit red cells conjugated with the antigen by the bis-diazo-benzidine ring (EA, RGG or Pic.Gp.SA) (Halpern, Jacob, Binaghi and Parlebas, 1961). Ileal strips and sera were taken on the 15th to 18th day after the sensitization.

The intensity of the delayed type of skin hypersensitivity reactions resulting from the intradermal injection of decreasing doses of the antigen (Pic.Gp.SA), 7 days (or more as indicated in the results) after sensitization, was evaluated by measuring the mean diameter of the lesions. The skin reactions began to appear 6–8 hours after the injection of the antigen and reached their full development 24 hours later.

RESULTS

I. Effect of the paralysis-inducing treatment with BSA on the anaphylactic sensitization with an unrelated antigen (EA or RGG)

The animals treated with BSA (800 mg./day) over a period of 15 days regularly developed an immunological paralysis towards this antigen. Negative results were observed in all three tests (Schultz-Dale reaction, passive cutaneous reaction, passive haemagglutination) for the serum anti-BSA antibodies, carried out at 10 days and at 2 months after the end of the treatment with BSA.

Alum precipitated ovalbumin (EA) or rabbit γ-globulin (RGG) (10 mg. i.p.) were injected at various intervals following the beginning of the administration of BSA. This treatment was always continued for at least 4 days after the sensitizing injection, in order to allow for persistence in the organism of the alum precipitated antigens. Results of these experiments are reported in Table 1.

When the sensitizing injection was performed on the same day as the beginning of the paralysis-inducing treatment, in spite of continuing this treatment for 10 more days, treated animals were sensitized to the same degree as control animals.

However, the longer after the beginning of the treatment the sensitizing injection was given the less sensitized the animals became. None of the animals became sensitized to

Table 1

Immunological reactions to ovalbumin (EA) and rabbit γ-globulin (RGG) in guinea-pigs paralysed with bovine serum albumin (BSA) (800 mg./day/animal)

Sensitizing antigen	*Route*	*Control animals*			*Animals treated with BSA (800 mg./day) injected with other antigens*											
					Day 1			*Day 5*			*Day 10*			*Day 15*			*Day 18*		
		*S.-D. reaction**	*PCA†*	*P. haem.‡*	*S.-D. reaction*	*PCA*	*P. haem.*	*S.-D. reaction*	*PCA*	*P. haem.*	*S.-D. reaction*	*PCA*	*P. haem.*	*S.-D. reaction*	*PCA*	*P. haem.*	*S.-D. reaction*	*PCA*	*P. haem.*
EA (Alum precipitated 10 mg.)	i.p.	+++	100	640		32		++	10	160	0	0	16						
		+++	256	5120		32		0	0	10	0	0	16						
		+++	128	2560		64		0	0	10	0	0	16						
		+++	64	640		16		0	0	10	0	0	16						
		+++	128	1280		128		+++	40	160	0	0	16						
								+++	40	320	0	0	16						
								+++	20	640									
	i.v.	++	32	640				0		10	0	0	16						
		+++	256	2560				0		10	0	0	32						
		+++	64	1280				+		40	0	0	16						
											0	0	8						
	s.c.	+++	128	4096							+++		1024	0	0	8	0		16
		++	32	2048							++		256	++	16	256	0		16
		+++	128	1024							++		512	+++	32	1024	0		8
														0	0	16	++		256
RGG (Alum precipitated 10 mg.)	i.p.	+++	128	2048	+++	128	1024	0			0	0	8						
		+++	64	2048	+++	64	1024	++			0	0	8						
		+++	256	1024	+++	64	2048	++			0	0	8						
		+++	128	1024	+++	128	1024	++		32	0	0	8						
		++	32	256	+++	64	1024	0		8									
								+		16									
	i.v.	++	40	1024				0	0		0	0	16						
		++	20	1024				++	5		0	0	8						
								++	10		0	0	8						
											0	0	16						
	s.c.	++	30	256				++	8		0	0	32				0	0	
		++	16	512				+++	32		+++	64	1024				++	4	
								+++	32		+++	32	1024				0	0	

* Schultz-Dale reactions; graded as follows: maximal (+++), moderate (++), minimal (+) and negative (0).
† Passive cutaneous anaphylaxis; reciprocal of the dilution of the serum giving a threshold reaction.
‡ Passive haemagglutination; expressed by the reciprocal of the haemagglutination titre.

the EA or RGG injected intraperitoneally on the 10th day of the paralysis-inducing treatment. The three tests for anaphylactic sensitization showed negative results (a haemagglutination titre below 1/16 seems not to be specific).

The same results were observed when the injection was given intravenously. However, when the sensitizing antigens were injected subcutaneously it took longer for the paralysing treatment to prevent sensitization. As is indicated in Table 1, only one animal injected with the sensitizing antigen before the 15th day of the paralysing treatment failed to be sensitized.

The inhibition of sensitization towards EA or RGG brought about as described above, seems to be permanent. Guinea-pigs tested 2 months after the injection of sensitizing antigens on the 10th day of the treatment showed neither Schultz-Dale reactions to EA or RGG, nor antibodies to these antigens. Furthermore, when such animals were injected again with the same antigen (RGG), 3 months after the first injection, they gave a typical primary immune response whereas animals not so treated gave a typical secondary response (Fig. 1, curves B and C).

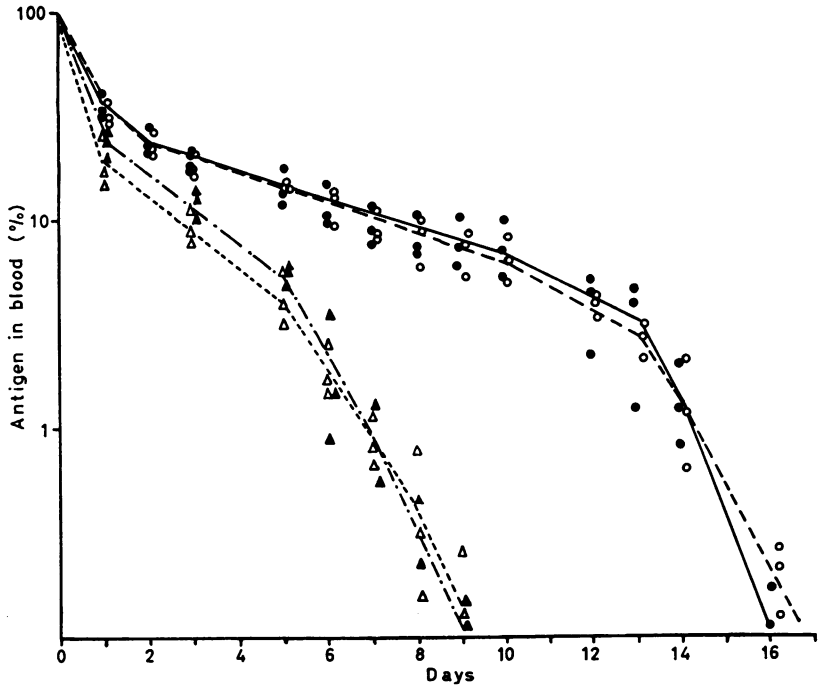

FIG. 1. Disappearance rate of the antigen (^{131}I RGG, 3 mg.) injected intravenously in: normal guinea pigs (curve A; ●——●), guinea-pigs previously injected with RGG on the 10th day of the paralysing treatment (curve B; ○— — —○), guinea-pigs previously sensitized with RGG (curve C; △- - - - -△) and guinea-pigs previously sensitized with RGG and given a paralysing treatment before the injection of ^{131}I RGG (curve D; ▲— · — · —▲).

These results show that immunological paralysis is specific only at the beginning of the BSA overloading treatment. As the series of paralysis-inducing injections is continued the immune unresponsiveness may spread to affect other protein antigens, unrelated to BSA.

Quantities of BSA Necessary to Induce Non-Specific Unresponsiveness

A study was undertaken to determine if such quantities of BSA (800 mg./day) are necessary to induce immunological unresponsiveness to ovalbumin. Groups of guinea-pigs were treated daily with various amounts of BSA (100, 200 and 400 mg./day) intraperitoneally and intravenously and injected i.p. on the 10th day of the treatment with 10 mg. of alum precipitated ovalbumin. As in previous experiments, treatment with BSA was continued for 4 days after the injection of ovalbumin.

As is recorded in Table 2, treatment of guinea-pigs with the above quantities of BSA is not sufficient to inhibit sensitization towards ovalbumin. However, in the treated animals, a significant decrease in the degree of anaphylactic sensitization is observed. Both Schultz-Dale reaction and passive haemagglutination showed a decrease in anti-EA antibody production as compared with the control animals. Furthermore, the larger the dose of BSA administered, the less anti-EA antibodies were produced.

TABLE 2

IMMUNOLOGICAL REACTIONS TO OVALBUMIN (EA) AND TO BOVINE SERUM ALBUMIN (BSA) IN GUINEA-PIGS TREATED WITH VARIOUS AMOUNTS OF BSA AND INJECTED WITH ALUM PRECIPITATED EA ON THE 10TH DAY OF TREATMENT

Animals	*Schultz-Dale reaction**		*Passive haemagglutination†*	
	EA	*BSA*	*EA*	*BSA*
Control	+++		2048	
	+++		4096	
	+++		4096	
Treated with BSA 100 mg./day	+	0	256	16
	++	+	512	64
200 mg./day	+	0	128	8
	+	0	128	8
	++	+	256	32
400 mg./day	0	0	32	8
	+	0	64	8
	0	0	16	8

* Graded as follows: maximal (+++), moderate (++), minimal (+) and negative (0).

† Expressed by the reciprocal of the haemagglutination titre.

In fact, the doses of BSA were not always sufficient to induce a specific immune paralysis (see Table 2). Moreover, as the animals were killed only 15 days after the end of BSA administration, it is possible that this time is not long enough to ensure elimination of the entire quantity of BSA injected, in order to permit the appearance of free anti-BSA antibodies in the circulation.

Duration of the State of Non-Specific Unresponsiveness

Guinea-pigs treated for 15 days with BSA (800 mg./day) were then injected with alum precipitated EA (10 mg. intraperitoneally), 8 days later. Results are given in Table 3. All these animals became sensitized as well as the control animals. Thus, the capacity of paralysed animals to be sensitized against another unrelated antigen is rapidly restored.

This is in contrast to specific immune paralysis which may last 3–6 months after cessation of the treatment, depending on the quantity of the antigen injected.

TABLE 3

IMMUNOLOGICAL REACTIONS TO OVALBUMIN (EA) IN GUINEA-PIGS TREATED WITH BOVINE SERUM ALBUMIN (BSA) (800 MG./DAY/ANIMAL) DURING 15 DAYS AND INJECTED WITH EA 8 DAYS AFTER DISCONTINUANCE OF THIS TREATMENT

Animals	*Schultz-Dale reaction**	*Passive haemagglutination†*
Control	+++	4096
	+++	1024
	+++	1024
Pre-treated	+++	1024
	+++	2048
	+++	1024
	+++	4096
	+++	2048

* Graded as follows: maximal (+++), moderate (++), minimal (+) and negative (0).
† Expressed by the reciprocal of the haemagglutination titre.

Effect of the Paralysis-Inducing Treatment on the Secondary Response to an Unrelated Antigen

Six guinea-pigs were sensitized with alum precipitated RGG (10 mg. intraperitoneally). Three months later the animals were divided into two groups: the animals of the first group were submitted to a paralysis-inducing treatment with BSA (800 mg./day) for 15 days. On the 10th day, these animals as well as animals of the second group (control) were given 3 mg. of ^{131}I-labelled RGG intravenously. The disappearance-rate of the labelled antigen from the blood was similar in treated and untreated animals: both groups responded with a disappearance-rate typical of a secondary response (Fig. 1, curves C and D). It is obvious that a paralysis-inducing treatment, administered under the above conditions, is without effect on a pre-established sensitization to an unrelated antigen.

II. EFFECT OF IMMUNE PARALYSIS INDUCED IN GUINEA-PIGS BY VARIOUS HETEROLOGOUS PROTEINS ON DELAYED HYPERSENSITIVITY TO CONJUGATED HOMOLOGOUS SERUM ALBUMIN (PIC.GP.SA)

A similar treatment was carried out on guinea-pigs with BSA (1000 mg./day intravenously and intraperitoneally) and, on the 15th day of this treatment, picrylated guinea-pig serum albumin mixed with complete Freund's adjuvant was injected (12·5 μg. of Pic.Gp.SA in 0·1 ml. of adjuvant) in each of the four pads. Treatment with BSA was continued for 4 days more. There was no inhibition of delayed hypersensitivity in the treated animals: delayed skin reactions in treated animals were of the same size as in control animals (Table 4, Exp. 1). Now, it is evident from Table 1, that when the sensitizing injection is given hypodermically while the paralysis-inducing treatment is performed intravenously and intraperitoneally, the period of pretreatment, necessary to inhibit sensitization towards an unrelated antigen, is considerably longer, by approximately 18–20 days. Consequently in another series of experiments we added to the usual daily treatment, local injections of paralysing antigens in the foot pads, given every other day.

Under these conditions, paralysis-inducing treatment with HSA by both systemic and local routes, provoked a definite repression of the delayed hypersensitivity, provided the treatment was initiated 10 days before the sensitization. When the pretreatment was reduced to 3 days, it had no influence on the size of the delayed reactions (Table 4, Exp. 2).

TABLE 4

EFFECT OF PARALYSING TREATMENT WITH DIFFERENT SERUM PROTEINS ON DELAYED-TYPE HYPERSENSITIVITY TO PICRYLATED GUINEA-PIG SERUM ALBUMIN (PIC.GP.SA)

Exp. No.	*Treatment with the paralysing antigen*					*Mean size (mm.) of skin reactions elicited by increasing concentrations of sensitizing antigen*			*No. of animals*
	Serum protein	*Route of injection and dose*		*Duration (days)*					
		I.V. (mg./24 hr.)	*Local* (mg./48 hr.)*	*Preceding sensitization*	*Following sensitization*	0·6 µg./0·1 *ml.*	2·5 µg./0·1 *ml.*	10 µg./0·1 *ml.*	
	0					6 ± 3·6	11 ± 3·3	17·9 ± 3·3	50
1	BSA	1000	—	15	4	5·5	11·3	16	7
2	HSA	600	140	3	7	7·7	13·5	19	2
		600	140	10	7	—	±	8·7	5
3	BGG	400	65	6	7	—	—	10	6
4	HGG	300	50	6	7	—	—	10	10
5	HGG	300	—	0	7	—	5·9	10·7	6
6	HGG	—	120†	3	7	—	±	7	4

* Injections were given subcutaneously in each foot-pad preceding sensitization The figures in the table indicate the total amount injected in the foot-pads.

† This dose is repeated every 24 hours.

Gamma-globulins, whether of bovine or human origin, appeared to be much more efficient in their inhibitory effect than albumins. BGG and HGG injected at half the amount of BSA or HSA and for shorter periods before sensitization produced approximately the same degree of inhibition as HSA (Table 4, Exp. 3 and 4). Furthermore HGG was effective when injected either by the systemic or by the local route alone (Exp. 5 and 6).

These results show that, during immune paralysis, a period of non-specific unresponsiveness may be observed, even if sensitization is performed using Freund's adjuvant.

Duration of the Inhibitory Effect Observed during Immune Paralysis on the Delayed Hypersensitivity to an Unrelated Antigen

Experiments were undertaken to study the duration of repression of delayed type of hypersensitivity produced by an immune paralysis-inducing treatment with HGG. In this series of experiments, treatment was initiated 7 days before sensitization and continued for 7 days afterwards with both systemic and local daily injections. HGG and Pic.Gp.SA were injected only in the two hind foot-pads. Under these conditions, a complete suppression of delayed hypersensitivity was observed when tested with Pic.Gp.SA on the 7th day following sensitization (Table 5). Even an intracutaneous injection of 100 µg. of Pic.Gp.SA produced no delayed reaction. Fourteen days after sensitization, i.e. 7 days after discontinuing the paralysing treatment, a certain degree of delayed hypersensitivity was evidenced, however, by cutaneous reactions to the highest doses of antigens. Twenty-one days after sensitization, the delayed reactions became more pronounced and, after 2 months, reached the same intensity as in the control animals (Table 5).

From the reported data, it can be inferred that the inhibition of delayed hypersensitivity induced during the immune paralysis to an unrelated antigen, is a transient phenomenon. Immunological response to the sensitizing antigen is inhibited during the paralysis-inducing treatment and then gradually appears, reaching the intensity of the control animals' hypersensitivity 2 months after discontinuance of the paralysing treatment.

In Table 6 are reported results of the dosages of circulating anti-Pic.Gp.SA antibodies during the above experiments. It is evident that antibody production to Pic.Gp.SA was definitely counteracted by the treatment with HGG; passive haemagglutination titres of treated animals were constantly lower than those of control animals. On the 7th day after sensitization, the haemagglutination titre in the two groups of animals was low because the antibody production at this time is at an early stage; the titre rapidly rose afterwards in control animals, whereas treated animals showed a slower increase of circulating antibodies, in spite of cessation of the treatment. Their level of haemagglutination titre was still lower at the end of the 2 months observation period.

TABLE 5

DURATION OF THE INHIBITORY EFFECT PRODUCED BY A TREATMENT WITH HGG DISCONTINUED ON THE 7TH DAY AFTER SENSITIZATION ON SUBSEQUENT DEVELOPMENT OF DELAYED-TYPE HYPERSENSITIVITY TO PIC.GP.SA

Days following sensitization	*Treatment**	*Mean size (mm.) of skin reactions elicited by increasing concentrations of sensitizing antigen*		
		0·6 µg./0·1 *ml.*	2·5 µg./0·1 *ml.*	10 µg./0·1 *ml.*
7	Control	10	12	16·5
		0	11	22
		0	16	20
		9·5	18	22·5
		7·5	12	18
		7	17·5	23·5
		10	10	22
	Average	6·3	13·8	20·6
	Treated	0	0	0
		0	0	0
		0	0	0
		0	0	0
	Average	0	0	0
14	Control	14·5	15·5	26
		15	12·5	21
		0	10	20
		12·5	18	20·5
		14	14	19·5
		0	12·5	17·5
		9·5	13	22·5
	Average	9·3	13·6	21
	Treated	0	0	6
		0	0	21·5
		0	0	10
		0	0	8
	Average	0	0	11·4

TABLE 5 (*contd.*)

Days following sensitization	*Treatment**	*Mean size (mm.) of skin reactions elicited by increasing concentrations of sensitizing antigen*		
		0·6 μg./0·1 *ml.*	2·5 μg./0·1 *ml.*	10 μg./0·1 *ml.*
21	Control	13 11·5	16·5 9·5	20·5 16·5
	Average	12·3	13	18·5
	Treated	0 0	7 0	16 3
	Average	0	3·5	9·5
60	Control	10·5 8 10 5 10 8 0	10 5 10 0 10 8 10	23·5 15 18 16·5 22·5 22·5 19
	Average	7·3	7·6	19·6
	Treated	13 5 0 9 0 3 3 14·5	8 10 0 10 6 5 4 10	20·5 19 12·5 19·5 11 6 14·5 26
	Average	6	6·6	16

* 300 mg./24 hours i.v. and 20 mg./24 hours in each hind foot-pad.

DISCUSSION

The afore-mentioned experiments show that immunological paralysis induced in adult guinea-pigs can, under certain conditions, involve an immune unresponsiveness towards antigens unrelated to the paralysis-inducing one. Indeed neither BSA and RGG, nor HSA, HGG or BGG and Pic.Gp.SA are known to cross-react. Weigle and McConahey (1962) recently demonstrated that BSA and EA do not cross-react either, and that the earlier reported cross-reactions between these two proteins (Dixon and Maurer, 1955b; Germuth, Pace and Tippett, 1955) are due to an impurity present in the ovalbumin preparations. Furthermore, the same author (Weigle, 1961) showed that only proteins cross-reacting by more than 30 per cent, can benefit from a reciprocal tolerance.

To obtain this non-specific suppression of both immediate and delayed hypersensitivity, two conditions appear to be necessary:

(a) To start the paralysis-inducing treatment several days before injecting the sensitizing antigen.

(b) To inject the sensitizing antigen under cover of the paralysis-inducing treatment, which must be continued for several days following the sensitizing injection.

TABLE 6

PASSIVE HAEMAGGLUTINATION TITRE (RECIPROCAL) OF SERA AT VARIOUS DAYS FOLLOWING SENSITIZATION OF GUINEA-PIGS TREATED WITH HGG AND SENSITIZED WITH PIC.GP.SA; ADMINISTRATION OF HGG DISCONTINUED THE 7TH DAY AFTER SENSITIZATION

Treatment	*Days following sensitization with Pic.Gp.SA*			
	7	14	21	60
Control	128	20480	51200	800
	16	20480	38400	100
	8	2560	38400	1600
	8	38400	38400	200
	64	12800		400
	256	51200		800
	16			400
Average	70·1	24320	41600	614
Treated	16	640	640	100
	4	2560	38400	200
	0	1280	12800	400
	8	1280	400	800
	8	6400	25600	800
	32	6400	6400	200
	8	1600		50
	16			50
Average	11·5	2880	14040	325

The need to provide for these conditions should explain why Dixon and Maurer (1955a), Felton *et al.* (1955) and Smith and Bridges (1958) failed to observe this phenomenon: Dixon and Maurer injected into rabbits a mixture of antigens (human plasma) right from the first day of the paralysis-inducing treatment, thus producing an immunological paralysis towards the major constituents of the plasma (albumin, γ-globulin) and an immune response towards the minor constituents (α- and β-globulins); Felton *et al.* and Smith and Bridges injected the sensitizing antigen too long after the injection of the paralysis-inducing one. In fact, sensitization occurred in our experiments, even if injection of the sensitizing antigen followed discontinuation of the paralysis-inducing treatment by as little as 8 days. Thus, Hanan and Oyama's (1954) observation that the immune response to ovalbumin is significantly diminished or absent in rabbits tolerant to BSA, can be explained by the fact that these authors injected ovalbumin during the treatment of rabbits with the BSA.

Why are these two conditions necessary to induce non-specific unresponsiveness?

The requirement that the injection of the sensitizing antigen should be performed on the 8th to 10th day following the beginning of the paralysis-inducing treatment may be explained by the necessity for the paralysing antigen to reach and effectively paralyse all the immunologically competent cells, before the arrival of the sensitizing antigen. This view accounts for the considerably longer time of pre-treatment required when injection of the sensitizing antigen is given by a route (subcutaneous) other than that of paralysis-inducing antigen (intravenous and intraperitoneal). This condition is similar to that observed during the inhibition of antibody production by X-rays, cortisone or 6-mercaptopurine. It is known that these agents are active only when employed before the antigen

injection (Taliafero and Taliafero, 1951; Berglund and Fagraeus, 1956; Sterzl, 1960). The fact that X-rays are active even when given 4 hours after antigen injection (Dixon, Talmage and Maurer, 1952) is not contradictory, for X-rays penetrate the whole organism instantaneously and affect competent cells before they are overtaken by the antigen. It appears therefore that these non-specific inhibitory agents, as well as the paralysis-inducing treatment, must act before or at least during the earliest stage of antigenic stimulation. This statement is further supported by the fact that a paralysis-inducing treatment with BSA has no effect on the secondary response to RGG (Fig. 1). It follows that non-specific unresponsiveness differs from specific immune paralysis by the fact that a previous sensitization could be overcome by paralysing treatment, using larger doses of the same antigen (Felton *et al.*, 1955; Hašek, 1962).

Specific immune paralysis induced with non-living antigens lasts several weeks or months according to the quantity injected (Smith, 1960). How then does one explain how an injection of the sensitizing antigen, when given only 8 days after the stopping of paralysis-inducing treatment, regularly results in sensitization of the animals (Table 3)?

The same question arises from the experiments in which delayed type hypersensitivity to Pic.Gp.SA appeared some days after discontinuing the paralysis-inducing treatment. Tests for delayed skin reactions in these animals on the 7th day were negative, whereas when animals were tested on the 14th day reactions were positive for the stronger concentrations of the antigen (Table 5). Data available at present are not sufficient to explain these facts. A simple explanation might be as follows: to obtain a non-specific unresponsiveness during immune paralysis, the local concentration of the paralysis-inducing antigen in close vicinity to the competent cells must be somewhat higher than that necessary to induce a specific unresponsiveness.

Our results show that:

(a) To induce non-specific unresponsiveness a dosage of BSA of 800 mg./day is necessary. In these conditions antibody production to ovalbumin is completely suppressed. A dose of 400–500 mg. BSA per day can induce only a partial unresponsiveness to ovalbumin (Table 2), though the specific paralysis (to BSA) is total. (See also Dixon and Maurer, 1955a.)

(b) In delayed hypersensitivity experiments, combination of local injections of the paralysing antigen with the systemic treatment appears necessary in order to produce any noticeable inhibition to the sensitizing antigen. In this way the concentration of the paralysis-inducing antigen in the regional lymph nodes, which accounts for the delayed hypersensitivity (Neveu, Biozzi, Halpern, Liacopoulos and Branellec, 1963) is considerably increased. Even under these conditions, hypersensitivity appears soon after discontinuing the treatment (Table 5) whereas anaphylactic sensitization is definitively inhibited when the paralysing treatment is continued for 5 days after sensitization. It is likely however, that in the later case, alum-precipitated antigen is metabolized in a few days, while the antigen emulsified in Freund's adjuvant persists for a longer period and therefore antigenic stimulation lasts beyond the period of 7 days for which treatment is prolonged.

On the other hand, the half-life of the proteins used as paralysis-inducing antigens is, in guinea-pigs, rather short: BSA 2·3 days, BGG 1·8 days and HGG 1·9 days (Weigle, 1960). Thus, 8 days after discontinuing the paralysing treatment there remains in the body of the treated animal less than a tenth of the quantity existing at the end of the

treatment. This amount of antigen, largely sufficient to maintain a specific unresponsiveness (though not to induce it), appeared to be inadequate to inhibit sensitization towards another non-related antigen.

The actual mechanism of the non-specific unresponsiveness during immunological paralysis cannot be known in the present state of knowledge on the formation of antibodies. Neither selective nor instructive theories allow a coherent explanation of the facts reported in this study. Nevertheless one should be able to link non-specific unresponsiveness with another phenomenon, equally unexplained by these theories: that of 'antigen competition'.

This fact, first observed by Glenny and Waddington (1926) seems to be confirmed by recent studies of Barr and Llewellyn-Jones (1953), Adler (1957), Abramoff (1960) and Abramoff, Zickes and Joyce (1961). These authors studied antibody production in guinea-pigs or chickens after injection of two antigens. They observed that according to the kind and dosage of these antigens and the timing of the injections, production of antibodies towards the one or the other of the two antigens together can be significantly reduced. In our experiments, we observed that when guinea-pigs, treated with amounts of BSA lower than necessary to induce an immune paralysis, are injected with ovalbumin, antibody production against EA as well as against BSA, is significantly decreased (Table 2).

This phenomenon seems to indicate that, when some of the competent cells are occupied with antibody production towards antigen A, a second unrelated antigen B is bound to meet with a smaller number of competent cells. Thus, so far as the antigens which have been studied are concerned, induction of non-specific unresponsiveness might have some analogies with competition of antigens: when all the competent cells of a treated animal are paralysed owing to the presence of an antigen in high concentration, the treated animal should not be able to respond to a second antigenic stimulation. This concept does not necessarily demand a uniformity of the immunological apparatus, but only a lack of very close specialization of the competent cells.

Our experiments provide some information on the relationship between anaphylactic sensitization and delayed hypersensitivity. Reported results show that a paralysis-inducing treatment with a protein can inhibit antibody production as well as delayed hypersensitivity to unrelated proteins. The studies of Uhr and Scharff (1960) and Celada and Carter (1962) on antibody formation and delayed hypersensitivity showed that both types of hypersensitivity are inhibited by X-irradiation of animals before sensitization. The doses of X-rays necessary to inhibit delayed hypersensitivity were, however, larger than those necessary to eliminate the antibody response. Comparing the results reported in Table 1 with those in Table 4, we should presume that antibody production is more easily reduced by the paralysis-inducing treatment than is delayed hypersensitivity. Notwithstanding these quantitative differences, the fact that such a treatment can inhibit the immediate type as well as the delayed type of hypersensitivity (to unrelated antigens) seems to indicate that the two types of hypersensitivity are dependent on mechanisms which might have some component(s), probably cellular, in common.

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