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STUDIES ON THE EFFECT OF SYSTEMIC ADMINISTRATION OF SENSITIZERS IN GUINEA-PIGS WITH CONTACT SENSITIVITY TO INORGANIC METAL COMPOUNDS

II. THE FLARE-UP OF PREVIOUS TEST SITES OF CONTACT SENSITIVITY AND THE DEVELOPMENT OF A GENERALIZED RASH

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SUMMARY

The intravenous injection of $K_2Cr_2O_7$ produces a specific flare-up of old sites of contact sensitivity to $K_2Cr_2O_7$ and, in very highly sensitive animals, there is also a generalized rash all over the body. A similar specific flare-up of old contact sensitivity site to BeF_2 can be produced by the intravenous injection of Be lactate.

Histological examination of the flare-up site shows an infiltration with polymorphonuclear leucocytes. Flare-up reactions can be blocked by an antiserum directed against polymorphonuclear leucocytes but not by anti-LNPF serum which blocks contact sensitivity reactions specifically. It is suggested that the flare-up reaction is not a cell-mediated immune reaction but could be similar to the Arthus reaction and is possibly mediated by humoral antibodies.

The histological appearance of the generalized rash is similar to that seen on examination of the rash produced by serum sickness in the guinea-pig. The rash can be produced by absorption of $K_2Cr_2O_7$ from an abraded area of skin as well as by intravenous injection.

INTRODUCTION

Sulzberger (1929) demonstrated that an intracardiac injection of neoarsphenamine to sensitive guinea-pigs would evoke a flare-up on the site of a previous intradermal skin test, which had already resolved. This was associated with the development of a generalized erythematous rash all over the body in a proportion of the animals. These results were

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confirmed by Kaplun & Moreinis (1930) though in a very small proportion of the animals tested. A similar flare-up of sites of previous contact reactions to dinitrochlorobenzene (DNCB) was observed by Frey, de Weck & Geleick (1964) in 54% of guinea-pigs sensitized to DNCB who had been given an intravenous injection of dinitrobenzene sulphonic acid ($DNBSO_3$). In these experiments the flare-up occurred maximally 48–72 hr after the injection of $DBNSO_3$. This was associated with a generalized erythema of the skin which appeared 5 hr after the injection of $DNBSO_3$ and persisted for some time with generalized scaling. This generalized eruption occurred regularly in highly-sensitized animals but was not seen in animals with only a low degree of sensitivity. de Weck *et al.* (1964) considered the specific flare-up to be a manifestation of cell-mediated immunity in which antigen arriving through the circulation reacts with residual specifically sensitive lymphocytes remaining at the site of an old contact reaction. They also considered that the generalized dermatitis which was seen in 20% of sensitive animals given $DNBSO_3$ intravenously was also a similar manifestation of cell-mediated immunity.

The demonstration that both a flare-up of previous contact reaction sites and a generalized erythematous rash could be observed in guinea-pigs sensitive to potassium dichromate ($K_2Cr_2O_7$) or beryllium fluoride (BeF_2) given respectively potassium dichromate or beryllium lactate intravenously, acted as a stimulus to look further into the mechanism of these two phenomena. The observation that the flare-up reaction histologically contained many polymorphonuclear leucocytes and occurred strongly as early as 6 hr after intravenous injection cast doubt on whether these reactions were in fact manifestations of cell-mediated immunity and suggested that experiments should be undertaken to determine whether there might be another mechanism than that which first produced the contact reaction, to account for its subsequent flare-up when antigen was administered systemically.

MATERIALS AND METHODS

The animals used were albino guinea-pigs of the Hartley strain as in the previous paper (Polák & Turk, 1968). Sensitization to $K_2Cr_2O_7$ and BeF_2 skin testing and intravenous injection of $K_3Cr_2O_7$ were also as described in the previous paper (Polák & Turk, 1968). In some experiments guinea-pigs were injected intravenously with 40 mg/kg chromium sulphate ($Cr_2(SO_4)_3$) in which chromium was in the trivalent as opposed to the hexavalent form. As before only strongly sensitive animals were used.

Preparation of antisera

All antisera were prepared in New Zealand White rabbits weighing between 2 and 3 kg.

Anti-guinea-pig polymorphonuclear leucocyte serum was prepared against guinea-pig polymorphs derived from an exudate produced in the peritoneal cavity by the injection of 3% bacteriological peptone as described by Humphrey (1955). The serum was absorbed first with an equal volume of guinea-pig erythrocytes, followed by a second absorption with $\frac{1}{4}$ volume of mixed spleen and mesenteric lymph node cells. The serum was heated at 56°C for 30 min before absorption.

Antiserum against a membrane free extract of guinea-pig lymph nodes (anti-lymph node permeability factor serum—anti-LNPF). This was prepared from pooled guinea-pig lymph nodes as described by Willoughby, Walters & Spector (1965).

All sera were sterilized by passage through a millipore filter before use.

The potency of anti-LNPF serum was assessed by its ability to block contact sensitivity to dinitrochlorobenzene (DNCB) completely. Five guinea-pigs were sensitized by the application of 0·02 ml 50% DNCB in acetone, 10 days later they were skin tested on the flank with 0·02 ml 0·5% DNCB in 2-ethoxyethanol. This produced strong red confluent skin reactions maximum 24 hr later. After a further 24 hr they were given 1 ml anti-LNPF intravenously and skin tested immediately afterwards with 0·02 ml 0·5% DNCB in 2-ethoxyethanol. All reactions were negative.

Production of rash due to serum sickness

Six guinea-pigs weighing between 300 and 350 g were given 2 ml normal rabbit serum intraperitoneally daily for 6 days. On the 7th day the skin was clipped and four out of the five animals showed a fine macular rash all over the trunk.

Preparation of tissues for histological examination

Skin from flare-up reactions on the site of a rash was taken from the anaesthetized animal before death and fixed in corrosive acetic fixative (95 parts of a saturated solution of $HgCl_2$ and 5 parts of glacial acetic acid) for 4 hr, then transferred to 70% ethanol. Following this it was processed in the normal manner, embedded in paraffin and sectioned at 5 μ. The sections were stained with haematoxylin and eosin.

EXPERIMENTAL RESULTS

Flare-up of previous sites of contact sensitivity

Table 1 shows the incidence of flare-up of contact sensitivity sites, induced 2 weeks previously and generalized rash following the intravenous injection of $K_2Cr_2O_7$. This incidence is expressed as a function of the dose of $K_2Cr_2O_7$ injected and the intensity of the original contact reaction. It can be seen that all guinea-pigs which had produced a strong reaction would show a flare-up of this reaction with a dose of 2 mg/kg $K_2Cr_2O_7$ or more given intravenously. Five out of seven strongly sensitized animals reacted with flare-up to a dose of 0·1 mg/kg given intravenously 2 weeks after the last skin test. However, flare-up reactions could not be produced when the dose was reduced to 0·01 mg/kg. Two out of eight animals sensitized with $K_2Cr_2O_7$ but which had failed to react with contact reactions were induced to produce a flare-up of the site of skin painting with 0·5% $K_2Cr_2O_7$ by the intravenous injection of 20 mg/kg $K_2Cr_2O_7$, indicating that this must have been the site of a subliminal reaction to the sensitizer. No flare-up was produced of sites of non-specific contact reactions to oxazolone or of the sites of intradermally induced delayed hypersensitivity reactions to tuberculin. No flare-up was produced at the site of repeated skin painting with 0·5% $K_2Cr_2O_7$ in non-sensitized animals. Flare-up reactions could be regularly produced in $K_2Cr_2O_7$ sensitized guinea-pigs by the intravenous injection of $Cr_2(SO_4)_3$ in which the chromium was injected in the trivalent as opposed to the hexavalent form used for sensitization.

Flare-up reactions were first seen as a faint pink blush at the site of previous contact reactions in some animals as early as 2 hr after intravenous injection of $K_2Cr_2O_7$. By 4 hr all animals showed a reaction and in some it was already fully developed. By 6 hr the flare-up reaction was completely developed in all animals. The reaction persisted for 24 hr, though weaker reactions could be seen to be on the wane. Traces of reaction were still

present 48 hr after injection but were resolving with slight scaling of the epidermis which lasted up to 1 week after injection.

In Table 2 it can be seen that flare-up reactions were most intense and occurred in all animals if induced 14 days after a previous contact reaction. The incidence of positive flare-up reactions and the intensity of such reactions diminished as the interval of the

TABLE 1. Proportion of sensitized animals showing flare-up of contact skin test site induced 2 weeks previously: (a) as a function of intensity of contact reaction and dose of $K_2Cr_2O_7$ injected intravenously, and (b) effect of 1 ml anti-LNPF serum given intravenously immediately before i.v. $K_2Cr_2O_7$

Dose of $K_2Cr_2O_7$ (mg/kg)	Intensity of contact reaction 2 weeks previously	Proportion of animals showing flare-up	Proportion of animals with generalized rash
20	0	2/8	0/8
20	+	9/10	0/10
20	++	20/20	6/20
10	++	7/7	0/7
2	++	7/7	0/7
0·1	++	5/7	0/7
0·01	++	0/7	0/7
20+1 ml anti-LNPF	++	6/6	0/6

One millilitre of anti-LNPF, given intravenously before contact testing completely blocked contact sensitivity to dinitrochlorobenzene.

TABLE 2. Intensity of flare-up reaction as a function of time between last epicutaneous contact and intravenous injection (expressed as percentage of animals tested—seven animals in all)

Time after last skin test	Intensity of flare-up		
	++	+	0
14 days	100·0	0	0
1 month	28·5	57·0	14·5
2 months	28·5	43·0	28·5
3 months	14·5	14·5	71·0

time between previous contact reaction and intravenous injection was increased from 14 days to 3 months. Intravenous injections of $K_2Cr_2O_7$ sooner than 14 days after skin test induced reactions no different from those produced at 14 days. Flare-up of contact reaction sites were seen in thirteen out of twenty-three animals sensitive to BeF_2 when 5 mg/kg Be lactate was injected intravenously 1 week after a positive skin reaction produced with 1% BeF_2 in 1% Triton X 100.

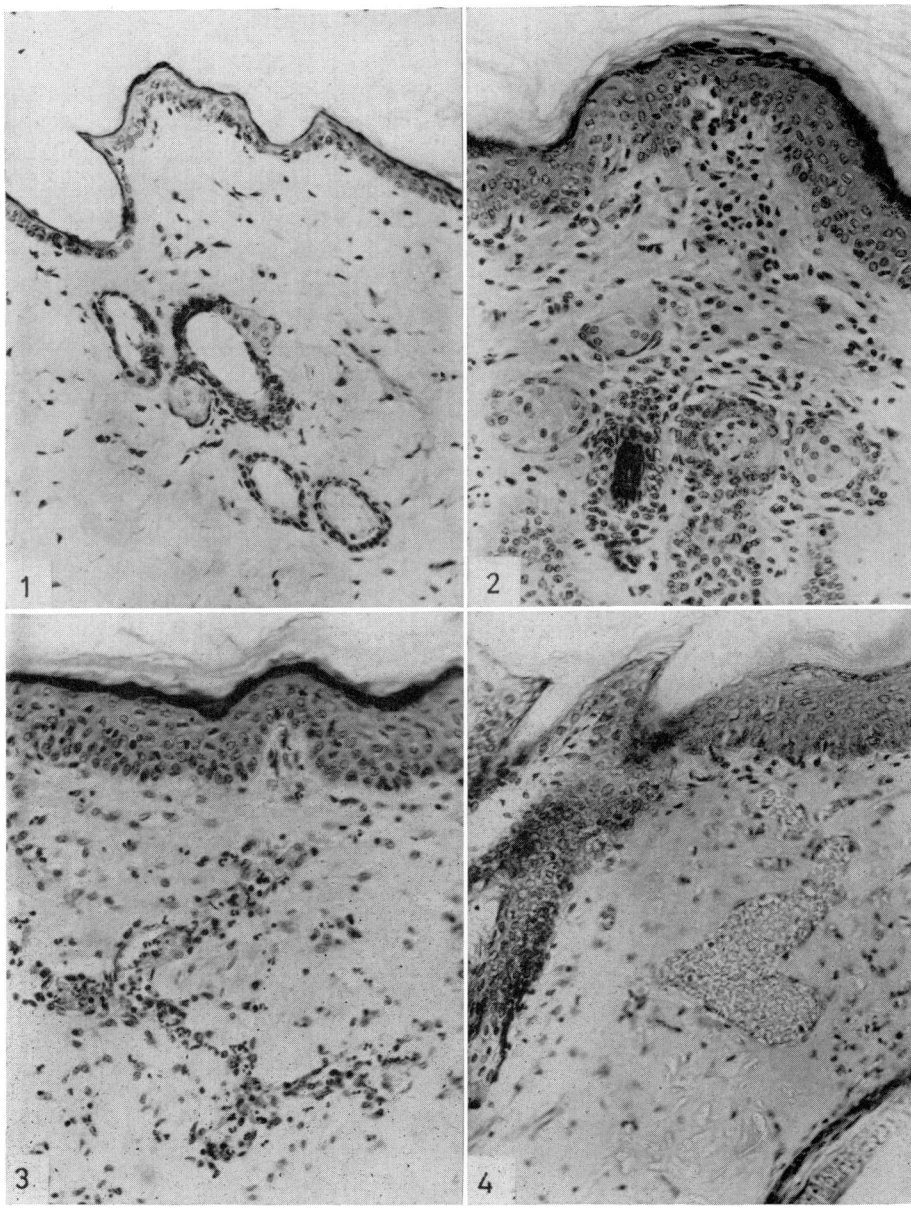

FIG. 1. Section of skin of epidermis of normal guinea-pig painted many times with $K_2Cr_2O_7$ in 1% Triton X 100. H & E, × 140.

FIG. 2. Section of skin, which had been the site of a contact sensitivity reaction to 0·5% $K_2Cr_2O_7$ 2 weeks previously. H & E, × 140.

FIG. 3. Section of skin which had been the site of a contact sensitivity reaction to 0·5% $K_2Cr_2O_7$ 2 weeks previously. Flare-up reaction 6 hr after intravenous injection of 20 mg/kg $K_2Cr_2O_7$. H & E, × 140.

FIG. 4. Section of skin through the papule of a rash 24 hr after the intravenous injection of 20 mg/kg $K_2Cr_2O_7$ into a guinea-pig sensitive to $K_2Cr_2O_7$. H & E, × 140.

Production of flare-up reaction of contact reaction sites in guinea-pigs subsequently made unresponsive

Five strongly sensitized guinea-pigs were injected intravenously with 20 mg/kg $K_2Cr_2O_7$ which produced a flare-up of a contact reaction induced 14 days previously. Twenty-four hours later they were skin painted with 0·5% $K_2Cr_2O_7$ in 1% Triton X 100 and failed to react. One week later they were given a second intravenous injection of 20 mg/kg $K_2Cr_2O_7$ and they produced a further flare-up of their first contact reaction site but not of the site of skin painting 1 week previously which had failed to produce a reaction. Twenty-four hours later they were skin painted on a third site with 0·5% $K_2Cr_2O_7$ in 1% Triton X 100 and failed again to produce a contact reaction. One week later they were given a third intravenous injection of 20 mg/kg $K_2Cr_2O_7$, they again produced a flare-up of the first contact site which had been that of a positive reaction, but failed to show any reaction in the site of the second and third contact sites which had been those of negative reactions.

Histological appearance of the flare-up reactions

Fig. 2 shows the picture of an old positive contact reaction site induced 14 days previously. It can be seen that there is still marked thickening of the epidermis as compared with Fig. 1 which is a picture of a section of skin of an unsensitized animal painted many times with 0·5% $K_2Cr_2O_7$ in 1% Triton X 100, the last time being 14 days before biopsy. There is also a marked infiltration of the tissue immediately under the epidermis with mononuclear cells. Fig. 3 is a picture of a similar area of skin at the peak of a flare-up reaction. There is dilatation of a capillary immediately under the epidermis and this is packed with polymorphonuclear leucocytes which are also to be seen migrating through the vessel wall into the surrounding tissue.

TABLE 3. Effect of anti-polymorphonuclear serum on flare-up reaction (1 ml or 2 ml of serum injected intraperitoneally 2 days before 20 mg/ml $K_2Cr_2O_7$ intravenously)

Animal No.	Polymorphonuclear leucocytes (per mm^3)	Lymphocytes (per mm^3)	Intensity of flare-up reaction
1	0	3048	0
2	0	1023	0
3	0	6162	0
4	0	2861	0
5	0	2150	0
6	0	7100	0
7	0	1225	+
8	0	5148	+
9	445	3560	0
10	5445	6550	++
11	1440	6930	+
12	230	1023	++
Controls			
1	3749	4319	++
2	3190	10150	++
3	7128	5940	++
4	2205	7670	++

Effect of anti-LNPF and anti-polymorphonuclear leucocyte serum on the flare-up reaction

One millilitre of anti-LNPF serum given intravenously immediately before the intravenous injection of 20 mg/kg $K_2Cr_2O_7$ failed to affect development of the flare-up of a contact reaction induced 14 days previously (Table 1). This serum was shown to be extremely potent in suppressing delayed hypersensitivity, in that it completely abolished contact reactions to DNCB when injected intravenously immediately before skin painting.

Anti-polymorphonuclear leucocyte serum in a dose of 1 or 2 ml was injected intraperitoneally 2 days before the intravenous injection of 20 mg/kg $K_2Cr_2O_7$. This treatment depleted the circulating polymorphonuclear leucocytes completely in eight out of twelve animals, without having much effect on the number of circulating lymphocytes. This treatment abolished the flare-up reaction completely in seven out of the twelve animals treated in this way and reduced the reaction in three others (Table 3).

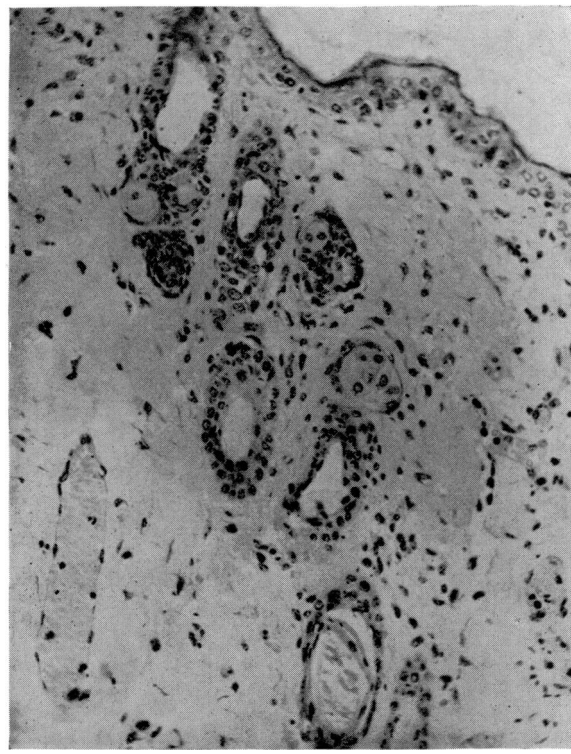

FIG. 5. Section of skin through a macule developed during serum sickness in a guinea-pig given 2 ml normal rabbit serum intraperitoneally for 6 days. Note dilated blood vessel without any perivascular infiltration as in Fig. 4. H & E, × 200.

The development of a generalized rash

Six out of twenty strongly sensitized animals developed a generalized erythematous rash when injected intravenously with 20 mg/kg $K_2Cr_2O_7$. This rash did not occur in weakly sensitized animals, nor in those injected with lower doses. Macroscopically the rash was a generally flat erythematous eruption though in one or two animals it had a definite papular

appearance. It started as faint pink spots between 6 and 8 hr after injection and became a definite red eruption by 24 hr. It generally remained unchanged for a further 24 hr and resolved with scaling over the next week. Histologically at 24 hr there was some thickening of the epidermis, and marked dilatation of superficial capillaries in the upper dermis, but without any obvious perivascular infiltration (Fig. 4). The appearance was not very different from that of the rash found in serum sickness in the guinea-pig (Fig. 5).

A rash could also be produced in strongly sensitized guinea-pigs by absorption of $K_2Cr_2O_7$ from the skin. Four strongly sensitive guinea-pigs were painted with 0·5% $K_2Cr_2O_7$ in 1% Triton X 100 on skin abraded previously with sandpaper. Twenty-four hours later there was a slight rash which was still visible after 72 hr but had resolved by 96 hr. Two weeks later the animals were skin tested for contact sensitivity by painting 0·5% $K_2Cr_2O_7$ in 1% Triton X 100 on undamaged skin. They reacted to this with only trace reactions. However, 2 days later when given 30 mg/kg $K_2Cr_2O_7$ intravenously a strong flare-up was produced of the sites of the contact painting which had only been observed as trace reactions. This was also associated with the development of a strong rash all over the body 24 hr after the intravenous injection of the $K_2Cr_2O_7$. Whether this was a flare-up of the site of the previous rash or the production of a new rash could not be determined.

No rashes were ever observed after the intravenous injection of 5 mg Be lactate in guinea-pigs sensitive to BeF_2. This was the highest dose which could be given without killing the animals.

DISCUSSION

Although the production of an immunologically specific flare-up of a previous contact sensitivity reaction and a generalized rash all over the body on systemic injection of a chemical sensitizer into sensitive animals has been known since the work of Sulzberger (1929), there has been no attempt to work out the mechanism of these reactions. Flare-up reactions could be produced in 70% of guinea-pigs with a dose as low as 0·1 mg/kg $K_2Cr_2O_7$, if the guinea-pigs were highly sensitive. Higher doses produced flare-up reactions in 100% of highly sensitized animals. The dose had to be dropped as low as 0·01 mg/kg intravenously to avoid getting a flare-up reaction.

It has been presumed that as the flare-up occurred at a site of previous cell-mediated immune reaction, both these reactions were also manifestations of cell-mediated immunity (de Weck, Frey & Geleick, 1964). No reports could be found in the literature of the histological appearance of these reactions. The finding that the flare-up reaction could be seen in some cases as early as 2 hr after intravenous injection and reached maximum intensity between 4 and 6 hr after intravenous injection, followed by the demonstration that the cellular infiltrate consisted mainly of polymorphonuclear leucocytes suggested that it might be possible that a mechanism other than delayed-type hypersensitivity might be involved. This impression was confirmed by the finding that if sensitized animals were made specifically unresponsive to further testing (Polák & Turk, 1968), a flare-up reaction could still be produced at the site of previous positive contact reactions. Further evidence that the flare-up reaction was not an example of cell-mediated immunity could be obtained by comparing the effect of anti-LNPF and anti-polymorphonuclear leucocyte serum on the reaction. It has been shown that anti-LNPF serum will block a number of immunological reactions in which cell-mediated immunity plays an integral part. These include contact

sensitivity in the guinea-pigs (Willoughby *et al.*, 1965), and pertussis hypersensitivity and allergic thyroiditis in the rat (Willoughby, 1966; Willoughby & Coote, 1966). Moreover, the effect of this serum appears to be specific in that it does not affect non-specific inflammation (Turk & Willoughby, 1967; Turk, Willoughby & Stevens, 1968). Anti-polymorphonuclear leucocyte serum on the other hand does not affect contact hypersensitivity in the guinea-pig (Inderbitzin, 1956), although it does block the Arthus reaction (Humphrey, 1955) and produces a 50–60% reduction in non-specific inflammation caused by the intradermal injection of turpentine (Turk *et al.*, 1968). The marked inhibition of the flare-up reaction by anti-polymorphonuclear leucocyte serum associated with a failure to affect this reaction with anti-LNPF serum, would indicate that it is more than likely that it develops as a result of a humoral antibody mediated mechanism. How this comes about can for the present only be a matter of speculation. It may be that the intravenous injection of sensitizer causes a release of a particular type of antibody into the circulation which then reacts with residual antigen present at the skin test site. An example of a mechanism similar to this is the recent demonstration by Pick & Feldman (1967) that the intravenous injection of tuberculin into a tuberculin sensitive animal will cause the release of an antibody-like substance into the circulation which when injected into a second animal confers reactivity of possibly an Arthus-type to tuberculin. Such a mechanism probably does not account for the flare-up reaction as this reaction only occurs at the site of a previous positive contact hypersensitivity reaction and does not occur at the site of skin painting in an unresponsive animal where such a reaction has not developed. However, a flare-up reaction will occur at the site of a subliminally positive reaction where there has probably been cellular infiltration without the development of erythema. Thus the flare-up reaction appears to need the presence of immunologically active cells still remaining at the site of the previous reaction. It is probable that the intravenously injected $K_2Cr_2O_7$ is converted to trivalent chromium which conjugates with a protein, forming an antigen (Samitz & Katz, 1964). Trivalent chromium in fact appears to be as effective as the hexavalent in producing the flare reaction. The antigen could then stimulate the production of humoral antibody by specific immunologically active cells at the site of a previous contact reaction which could react with residual antigen in the skin. Another possibility is that such a circulating antigen could react with small amounts of antibody being produced locally all the time under the stimulus of trace amounts of antigen in the skin which is, however, in insufficient concentration to cause an allergic reaction of its own accord. Experiments are in progress to elucidate the mechanism of this reaction further.

The type of flare-up reaction discussed above is probably different from the spontaneous flare-up of the site of original application or injection of sensitizer into the skin. This occurs as early as 4 days after first application of a chemical sensitizing agent to the skin in the case of oxazolone or between the 6th and 10th days after the intradermal injection of 150 μg of neoarsphenamine to cause sensitization. Such reactions have not been examined histologically in the experimental animal. However, they have been examined in man (Skog, 1966) and found to be associated with a mainly mononuclear cell infiltrate. A further type of flare-up reaction has also been observed following the intravenous injection of neoarsphenamine within 24 hr of a positive skin reaction to this compound produced by intradermal testing. This reaction is definitely different from the one we have described, as it is associated with marked haemorrhage and is thought to be a manifestation of the Schwartzman phenomenon (Frey & de Weck, 1967 personal communication). This type

of reaction is not observed following the injection of $K_2Cr_2O_7$ within 24 hr of a positive contact reaction to $K_2Cr_2O_7$.

The mechanism of production of the generalized rash is more difficult to elucidate. Such a rash can be produced as readily by the absorption of sensitizer, or the antigen formed by conjugation with skin protein, from the skin, as by intravenous injection. Histologically the most and somewhat unexpected finding is that such a rash is caused by superficial capillary dilatation without any obvious perivascular cellular infiltrate. Such an appearance is not very different from that found in serum sickness which is believed to be due to the circulation of immune complexes formed between antigen and antibody. It may be that such immune complexes cause the local release of pharmacological agents such as vasoactive kinins. Small amounts of bradykinin are in fact known to cause dilatation without perivascular cellular infiltration (Lewis, 1961).

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