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

III. THE EFFECT OF IMMUNOSUPPRESSIVE AGENTS ON ENHANCING THE UNRESPONSIVENESS OF ALREADY SENSITIZED ANIMALS AND AN INVESTIGATION OF THE ACTION OF THE EPICUTANEOUS TEST WITHIN TWENTY-FOUR HOURS

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SUMMARY

Permanent specific immunological unresponsiveness can be produced in guinea-pigs already sensitive to $K_2Cr_2O_7$ by the combination of an intravenous injection of 20 mg/kg $K_2Cr_2O_7$ and an epicutaneous contact with $K_2Cr_2O_7$ within 24 hr; 2 mg/kg $K_2Cr_2O_7$ produces only temporary desensitization. Methotrexate and cyclophosphamide for 1 week at the time of the intravenous injection increased the intensity of the suppression but did not prolong the unresponsiveness. Anti-thymocyte serum alone had no effect. A combination of cyclophosphamide and anti-thymocyte serum for 1 week at the time of the intravenous injection prolonged the unresponsiveness for 2 months, but the animals regained sensitivity by 3 months. The addition of prednisolone to this combination, reduced the duration of unresponsiveness produced by a combination of the specific sensitizer, cyclophosphamide and anti-thymocyte serum.

In animals given 20 mg/kg $K_2Cr_2O_7$ intravenously, removal of the site of epicutaneous application of $K_2Cr_2O_7$ within 24 hr inhibits the development of specific unresponsiveness. Histological examination of the site of application of the sensitizer reveals microscopic evidence of an inflammatory reaction, although no macroscopic changes can be seen. These findings are discussed in the context of the mechanism of the production of specific immunological tolerance in animals already sensitive.

INTRODUCTION

It has been shown previously (Polák & Turk, 1968a) that an intravenous injection of 20 mg/kg of potassium dichromate, in guinea-pigs which have been already sensitized to that antigen, produced a state of specific immunological unresponsiveness. The duration

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of this state of unresponsiveness was dependent on the time of the subsequent skin test. In cases when the test was applied up to 24 hr after the intravenous injection the animals became permanently desensitized, i.e. tolerant. However, the same dose of antigen followed by an epicutaneous test later than 24 hr (4 days, 14 days and 3 months) produced only a temporary state of unresponsiveness. The state of permanent desensitization was also dependent on the dose of antigen injected intravenously. It was found that a permanent state of unresponsiveness could only be produced with a dose as high as 20 mg/kg $K_2Cr_2O_7$. This dose was, however, in the range of LD 50 and, therefore, of no practical value in the therapy of chromium hypersensitivity. Smaller doses produced only a temporary desensitization and sometimes in only a proportion of the animals.

From these findings it was considered necessary to investigate whether it might be possible to convert the temporary desensitization produced by an intravenous injection of only 2 mg/kg $K_2Cr_2O_7$ into a permanent state of unresponsiveness by combining this injection with the administration of immunosuppressive agents. It was also important to examine the function of the relatively small amount of chromium painted on the skin 24 hr after the intravenous injection.

It is possible that a small dose of $K_2Cr_2O_7$ injected intravenously, produces only a temporary state of unresponsiveness by inactivating the circulating sensitized cells, without affecting the ability of the central lymphoid tissue to regenerate new sensitized cells. If this dose was combined with a course of immunosuppressive agents, it might be possible to suppress the ability of the central lymphoid cells to regenerate new sensitized cells, necessary for sensitivity to recover.

In recent work on contact sensitivity three agents have been shown to have a marked immunosuppressive effect in the guinea-pig, by blocking the ability of sensitized lymphocytes to proliferate in the central lymphoid organs. These agents are cyclophosphamide, methotrexate and anti-lymphocyte serum (Turk & Stone, 1963; Turk & Willoughby, 1967). An attempt was made, therefore, to see whether these agents either alone or together might prolong the state of unresponsiveness produced by an intravenous injection of a dose as low as 2 mg/kg $K_2Cr_2O_7$.

As, in the previous study, it was found that an epicutaneous test within 24 hr of the intravenous injection was also necessary to produce a prolonged state of unresponsiveness, an attempt was made to study what was the action of this application. Thus the skin test site was removed 6, 12 and 24 hr after painting to see whether this would affect the length of the period of unresponsiveness and histological examination was made of the sites to see whether any histological changes occurred in the skin, despite the absence of a macroscopic reaction, which might account for its action.

MATERIALS AND METHODS

Animals

Albino guinea-pigs of the Hartley strain, bred in the Institute of Dermatology, were used, weighing between 450 and 500 g. They were fed on pelleted diet RGP (E. Dixon and Son, Ware, Herts) liberally supplemented with fresh greens and hay.

Sensitization and skin testing

Guinea-pigs were sensitized to $K_2Cr_2O_7$ and skin tested as described previously (Polák & Turk, 1968a).

Induction of unresponsiveness

$K_2Cr_2O_7$ was injected intravenously through the marginal vein of the ear in doses of 20 or 2 mg/kg. Only strongly sensitive animals were used and the intravenous injection was followed by a first skin test 24 hr after injection.

Immunosuppressive agents

(i) *Methotrexate* sodium made by Lederle Laboratories Division of American Cyanamid Company, Pearl River, New York, U.S.A. was kindly given by Lederle Laboratories, a division of Cyanamid of Great Britain Ltd. Guinea-pigs were injected with this material in a dose of 10 mg/kg daily, intraperitoneally for 6 days.

(ii) *Cyclophosphamide* 'Endoxana' was kindly given by Ward Blenkinsop and Co. Ltd, London. It was injected in a dose of 20 mg/kg daily, intraperitoneally for 6 days.

(iii) *Prednisolone* phosphate disodium (Merck, Sharp and Dohme Ltd) was injected intraperitoneally in a dose of 4 mg/kg daily also for a period of 6 days.

(iv) *Anti-thymocyte serum* (*AThS*). Rabbits were injected intravenously with 10^9 washed fresh guinea-pig thymus cells on two separate occasions with an interval of 2 weeks. They were bled out 1 week after the last injection. The serum was absorbed with an equal volume of homologous erythrocytes and sterilized by passage through a millipore filter before use. Guinea-pigs were injected daily, intraperitoneally with 1 ml of this serum for 6 days.

All immunosuppressive agents were given in a 6-day course starting 2 days before the intravenous injection of the $K_2Cr_2O_7$.

Removal of skin test site

The skin test site produced by the epicutaneous application of 0·5% $K_2Cr_2O_7$ in 1% Triton X100 24 hr after the intravenous injection of 20 mg/kg $K_2Cr_2O_7$, was removed 6, 12, 24 and 48 hr after the application. The test site was removed under ether anaesthesia 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, and methyl-green–pyronin.

EXPERIMENTAL RESULTS

Prolongation of immunological unresponsiveness produced by an intravenous injection of 2 mg/kg $K_2Cr_2O_7$, by the administration of immunosuppressive agents

(i) *The effect of methotrexate alone* (Table 1, Fig. 1). A course of methotrexate started 3 days before skin testing produced no decrease in the ability of animals to manifest contact reactions to $K_2Cr_2O_7$. However, it prevented the development of a flare-up reaction of previous skin test sites (Polák & Turk, 1968b) in two out of nine animals following the intravenous injection of $K_2Cr_2O_7$. In the dosage used it did not prolong the unresponsiveness of the animals, but increased the degree of suppression of the skin reactions while the animals were on the drug. The dose of methotrexate used was of the order of the LD 50 for this drug and, therefore, use of this compound was discontinued.

(ii) *The effect of cyclophosphamide alone* (Table 1, Fig. 2). Cyclophosphamide started 3 days before skin test also produced no decrease in the ability of the animals to manifest

TABLE 1. Unresponsiveness of animals previously sensitized to potassium dichromate, following an intravenous injection of 2 mg/kg potassium dichromate, followed by an epicutaneous test within 24 hr, in guinea-pigs treated with immunosuppressive drugs

Drugs	Proportion of animals showing skin reactivity after:						
	Days				Months		
	1	3	7	14	1	2	3
Methetrexate	0/9	5/9	7/8	4/5	5/5		
Cyclophosphamide	0/9	0/9	7/9	8/8	8/8		
AThS	3/5	5/5	5/5				
Cyclophosphamide + AThS	1/10	1/10	1/8	1/8	3/8	3/8	8/8
Cyclophosphamide + AThS + prednisolone	0/13	0/13	4/11	4/11	4/7	3/3	

AThS, Anti-thymocyte serum.

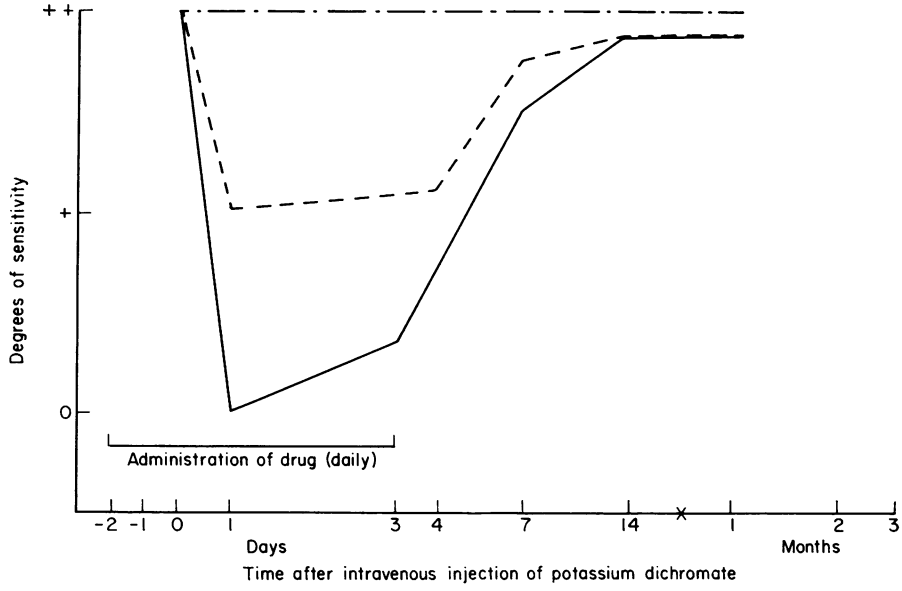

FIG. 1. Unresponsiveness of potassium dichromate sensitized guinea-pigs by intravenous injection followed by epicutaneous painting. ——, Methotrexate + $K_2Cr_2O_7$; — · —, effect of methotrexate, 10 mg/kg; - - - - -, intravenous injection of $K_2Cr_2O_7$, 2 mg/kg alone.

contact reactions to $K_2Cr_2O_7$. It prevented the development of a flare-up reaction of previous skin test sites following the intravenous injection of $K_2Cr_2O_7$. It prolonged the unresponsiveness of the animals slightly and this prolongation could be shown (Fig. 2) to persist for about 1 week after cessation of drug treatment. The compound was used in a dose below that of the LD 50.

(iii) *The effect of anti-thymocyte serum alone* (Table 1, Fig. 3). Anti-thymocyte serum (AThS) started 3 days before skin testing surprisingly did not suppress the contact reaction to $K_2Cr_2O_7$. It prevented the flare-up reaction in only one animal out of five, and in the dosage used had no effect on the degree of unresponsiveness produced by the intravenous injection of 2 mg/kg $K_2Cr_2O_7$.

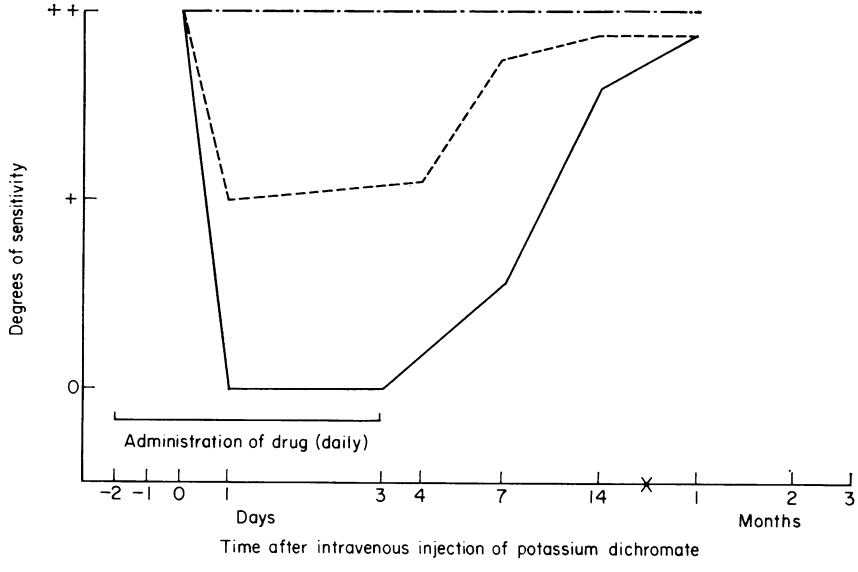

FIG. 2. Unresponsiveness of potassium dichromate sensitized guinea-pigs by intravenous injection followed by epicutaneous painting. ——, Cyclophosphamide+$K_2Cr_2O_7$; — . —, effect of cyclophosphamide, 20 mg/kg; – – – –, intravenous injection of $K_2Cr_2O_7$, 2 mg/kg alone.

(iv) *The effect of a combination of cyclophosphamide and anti-thymocyte serum* (Table 1, Fig. 4). A combination of cyclophosphamide and AThS started 3 days before skin testing produced a slight diminution in the intensity of the reaction of sensitized guinea-pigs to $K_2Cr_2O_7$. The flare-up reaction was blocked in six out of ten animals. This combination produced a marked prolongation of the degree of unresponsiveness of the animals to contact with $K_2Cr_2O_7$ which persisted for 2 months after the intravenous injection of $K_2Cr_2O_7$ covered by the two immunosuppressive agents. However, there was a sharp return of responsiveness 1 month later.

(v) *The effect of a combination of cyclophosphamide, anti-thymocyte serum and prednisolone* (Table 1, Fig. 5). Prednisolone was added to the previous combination in an attempt to obtain better results. However, the effect of adding this compound to the regime was to reduce the duration of the unresponsiveness that was obtained with cyclophosphamide and anti-thymocyte serum alone.

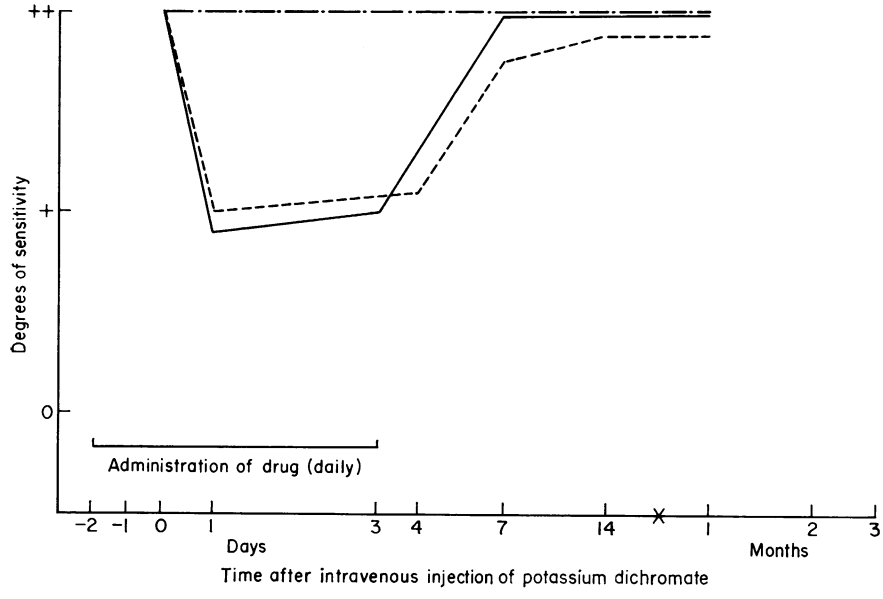

FIG. 3. Unresponsiveness of potassium dichromate sensitized guinea-pigs by intravenous injection followed by epicutaneous painting. ——, AThs+$K_2Cr_2O_7$; — · —, effect of AThS, 2 ml/kg; - - - - -, intravenous injection of $K_2Cr_2O_7$, 2 mg/kg alone.

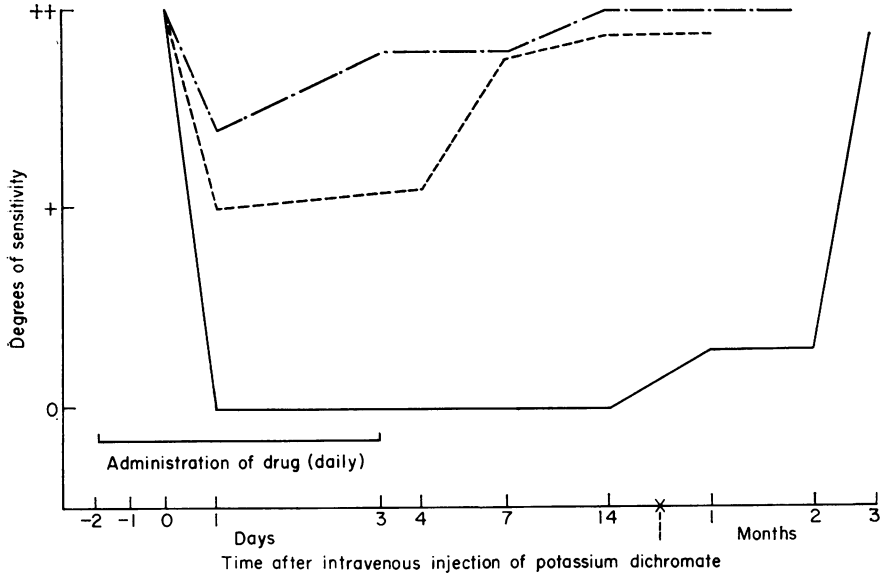

FIG. 4. Unresponsiveness of potassium dichromate sensitized guinea-pigs by intravenous injection followed by epicutaneous painting. ——, AThS+cyclophosphamide+2 mg/kg $K_2Cr_2O_7$; — · —, effect of a combination of AThS (2 ml/kg) and cyclophosphamide (20 mg/kg); - - - - -, intravenous injection of $K_2Cr_2O_7$, 2 mg/kg alone.

Effect of removing the skin test site, necessary for the development of prolonged immunological unresponsiveness (Table 2, Fig. 6)

In these experiments guinea-pigs, strongly sensitive to $K_2Cr_2O_7$, were injected intravenously with 20 mg/kg $K_2Cr_2O_7$ followed within 24 hr by an epicutaneous test with 0·5%

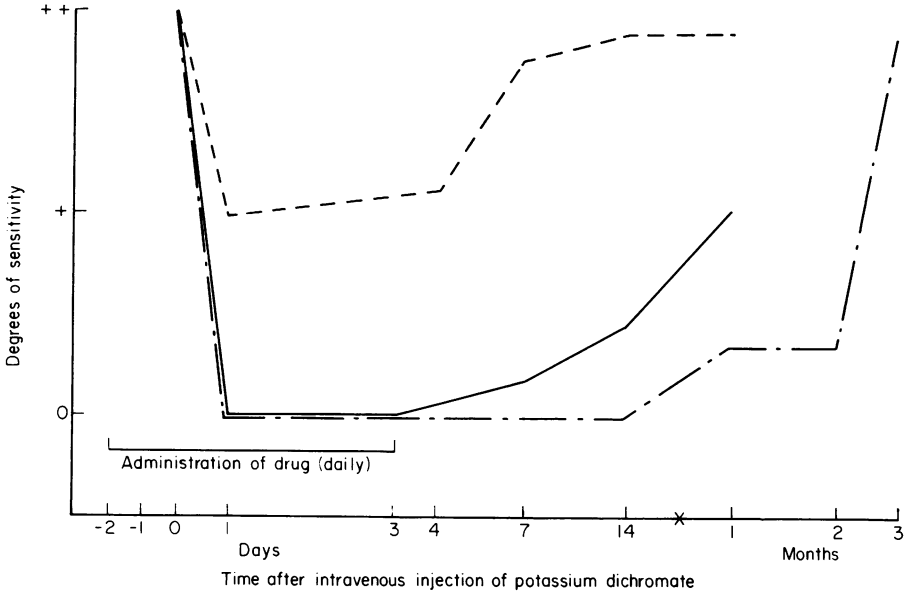

FIG. 5. Unresponsiveness of potassium dichromate sensitized guinea-pigs by intravenous injection followed by epicutaneous painting. ——, AThS (2 ml/kg)+cyclophosphamide (20 mg/kg)+prednisolone (4 mg/kg)+$K_2Cr_2O_7$ (2 mg/kg); — . —, AThS+cyclophosphamide +$K_2Cr_2O_7$; – – – –, intravenous injection of $K_2Cr_2O_7$ (2 mg/kg) alone.

TABLE 2. Unresponsiveness of animals previously sensitized to potassium dichromate, following an intravenous injection of 20 mg/kg potassium dichromate, followed by an epicutaneous test within 24 hr, if the epicutaneous test site is removed 6, 12 and 24 hr after testing

Time of removal of skin test site	Proportion of animals showing skin reactivity after:				
	Days		Months		
	1	14	1	2	3
6 hr	0/6	4/6	4/6	5/6	5/6
12 hr	0/6	4/6	4/6	5/6	5/6
24 hr	0/6	1/6	2/6	2/6	1/6

$K_2Cr_2O_7$ in 1% Triton X100. This has been found previously (Polák & Turk, 1968a) to produce complete unresponsiveness for at least 6 months. The skin test site 24 hr after the

intravenous injection, without which prolonged unresponsiveness does not develop, was removed 6, 12 and 24 hr after the application of the contact agent to the skin. Removal of the skin test site at 6 and 12 hr after application prevented the development of prolonged unresponsiveness, in that the animals began to react within 14 days after the intravenous injection and became progressively more sensitive during the ensuing 3 months. However, if the skin test site was left for 24 hr only two out of six animals showed skin reactivity in the next 3 months and these reactions were very weak in intensity.

Histological appearance of the skin test sites

Despite the fact that there was no macroscopic changes in the skin painted with $K_2Cr_2O_7$ in a sensitized animal 24 hr after the intravenous injection of 20 mg/kg $K_2Cr_2O_7$, the

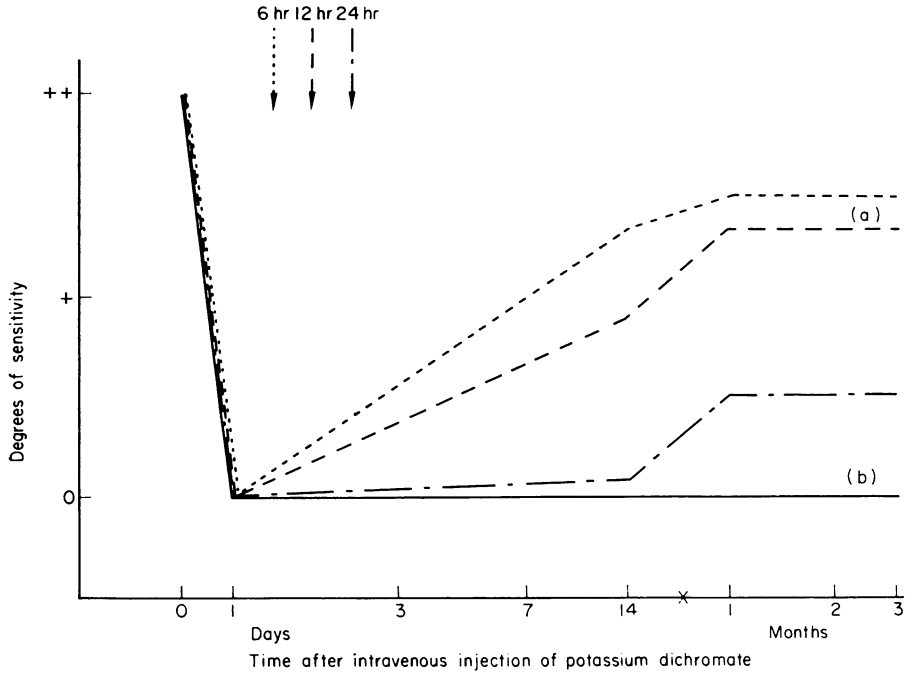

FIG. 6. Unresponsiveness of potassium dichromate sensitized guinea-pigs by intravenous injection followed by epicutaneous painting. (a) Effect of removal of skin test site 6, 12 and 24 hr after painting (24 hr after intravenous injection) + intravenous injection (20 mg/kg). (b) Intravenous injection of $K_2Cr_2O_7$ (20 mg/kg) without removal of skin test site.

histological changes were striking. As early as 6 hr there was infiltration of the dermis immediately under the epidermis with mainly mononuclear cells (Fig. 7a). By 12 hr the degree of infiltration had increased and the epidermis already showed acanthotic changes. At 24 hr the infiltration was at its maximum (Fig. 7b) and this was not found to diminish 48 hr after skin test. The infiltration was mainly with mononuclear cells although there were occasional polymorphonuclear leucocytes at 12 and 24 hr. Despite the fact that there was no macroscopic erythema, as early as 12 hr after skin test there was some dilatation of the superficial capillaries at the dermo-epidermal junction and this was marked at 24

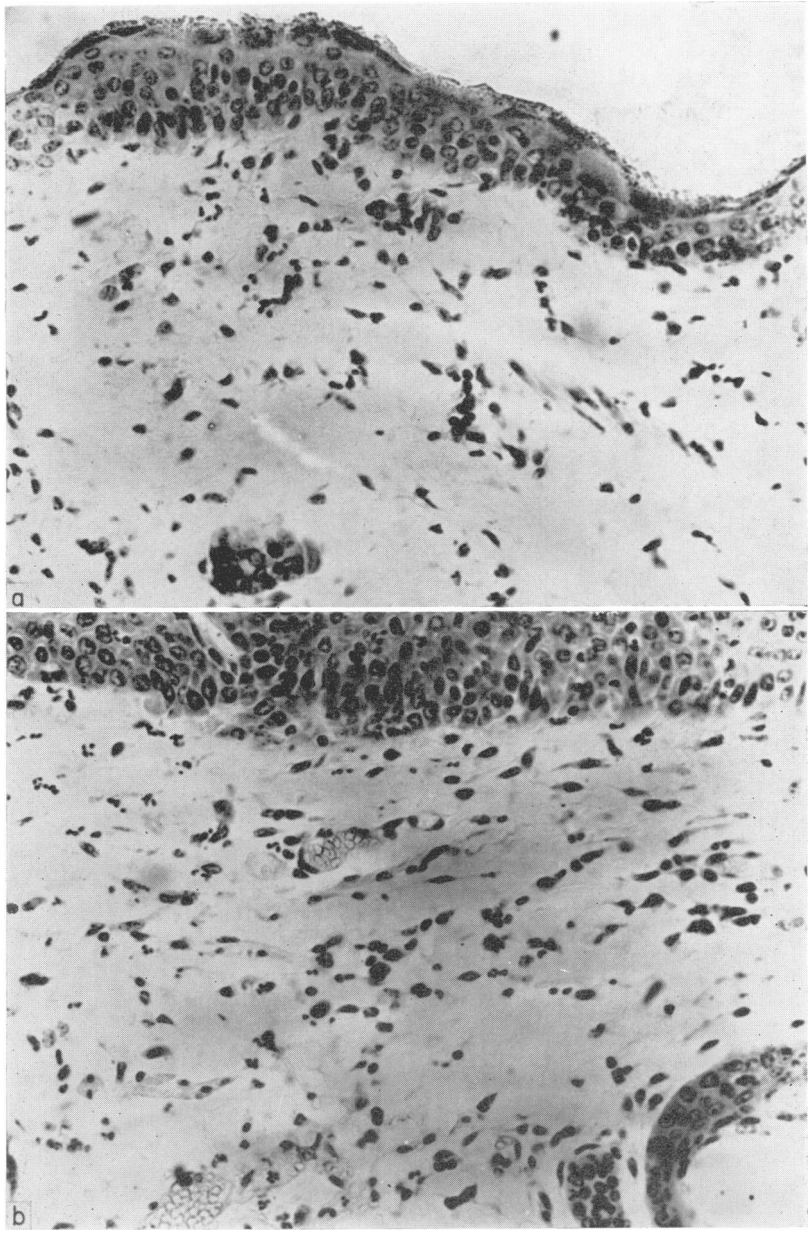

FIG. 7. Histological appearance of skin test 24 hr after intravenous injection. (a) Six hours after epicutaneous application of $K_2Cr_2O_7$. Scanty subepidermal infiltrate. (b) Twenty-four hours after epicutaneous application of $K_2Cr_2O_7$. Increased subepidermal infiltrate, dilatation of superficial subepidermal capillaries and acanthosis. H & E, × 250.

and 48 hr after skin test, as also was the degree of acanthosis in the epidermis. These changes were not seen after an epicutaneous test in non-sensitized animals, nor after intravenous injection of 20 mg/kg $K_2Cr_2O_7$ in the same sensitized animals in areas which had not been skin tested. It should be noted that the animals from which the skin test sites were biopsied did not develop generalized rashes as is seen occasionally after the intravenous injection of $K_2Cr_2O_7$ in specifically sensitized animals.

DISCUSSION

To produce permanent immunological unresponsiveness in already sensitized animals it is necessary to suppress the proliferation of specifically immunologically active cells in the central lymphoid tissues as well as inhibit the reactivity of these cells already in the circulation. It appears that this can be done in guinea-pigs sensitive to potassium dichromate (Polák & Turk, 1968a) or neoarsphenamine (Frey, de Weck & Geleick, 1966) by the intravenous injection of a large dose of the specific sensitizer followed by a dermal application of a small amount of the same agent within 24 hr. Permanent unresponsiveness cannot be obtained if the dose of the chemical substance injected intravenously is reduced, nor if the first skin test given after the intravenous injection is delayed longer than 24 hr.

In the present series of experiments it has been shown that prolonged unresponsiveness for 2 months can be obtained with a suboptimal dose of $K_2Cr_2O_7$ (2 mg/kg) if this is covered by a course of cyclophosphamide and anti-thymocyte serum, neither of which alone were able to prolong the period of unresponsiveness significantly. It is known that both these agents can act on the central lymphoid tissues to prevent the proliferation of lymphocytes during the period of sensitization between first contact with antigen and the development of a state of generalized sensitivity (Turk & Stone, 1963; Turk & Willoughby, 1967). Thus it is likely that these agents can act on the proliferating lymphocytes in the central lymphoid tissues to make them more susceptible to the tolerogenic effect of a lower dose of $K_2Cr_2O_7$.

The effect of prednisolone in reducing the degree of unresponsiveness produced by a combination of the specific sensitizer, cyclophosphamide and anti-thymocyte serum is interesting. A similar effect has been described by Simmons, Ozerkis & Hoehn (1968) who found that when cortisone was given before and during the administration of anti-lymphocyte serum in mice there was a reduction in the degree of immunosuppression of allograft rejection compared with when anti-lymphocyte serum was given alone. These authors have suggested that cortisone acts by blocking the mitogenic effect of anti-lymphocyte serum, which might be related to the immunosuppressive action of ALS. However, in other experiments Levey & Medawar (1966) have demonstrated that hydrocortisone can actually potentiate the effect of ALS. Moreover, there is no evidence that the mitogenic effect of ALS which occurs *in vitro* in the absence of complement occurs *in vivo* (Woodruff, 1967). All evidence to date actually points to a depletion of lymphocytes from those areas of the lymphoid tissue involved in the cell-mediated immune response, especially in the guinea-pig (Turk, Willoughby & Stevens, 1968). It is suggested that ALS acts directly suppressing the lymphocytes in the recirculating pool of long lived lymphocytes involved in the cell-mediated immune response (Denman, Denman & Embling, 1968). It would be more likely that prednisolone could act by inhibiting this action which is probably a cytotoxic effect of the serum.

The second necessity to produce a prolonged state of specific unresponsiveness in guinea-pigs already sensitized to $K_2Cr_2O_7$ is an epicutaneous test within 24 hr. One of the possibilities which has been suggested (Polák & Turk, 1968a) is that following the intravenous injection of the $K_2Cr_2O_7$ a small number of cells still remained which are only susceptible to conjugates formed between the sensitizer and the skin. It appears from the present experiments that the skin test site is necessary for at least 24 hr to produce the state of permanent unresponsiveness. Moreover, this is the site of a microscopic inflammatory reaction although there is no evidence of macroscopic changes in the skin. There appears to be a marked infiltration of the skin test site with mononuclear cells, presumably reacting with the chemical sensitizer conjugated to the skin. As a result of this there is marked vasodilatation of the superficial capillaries of the dermis and even some slight infiltration with polymorphonuclear leucocytes. There is also sufficient inflammatory stimulus to cause acanthosis in the epidermis. This could indicate that a reaction was occurring between sensitized cells and antigen at this site which presumably might result in the inactivation of these cells.

In experiments with *p*-nitrosodimethylaniline (NDMA) it has been shown that the application of this sensitizer, in the skin alone, can set in motion at the same time two phenomena, contact sensitization and unresponsiveness to further contact sensitization with the same compound (Lowney, 1967). Evidence thus exists that an epicutaneous test can act alone under certain circumstances to produce unresponsiveness. Thus there is no reason why in the $K_2Cr_2O_7$ system the epicutaneous application of the sensitizer should not be acting synergistically with the intravenous injection to produce the state of unresponsiveness observed. It is probable that the intravenous injection of hexavalent chromium ($K_2Cr_2O_7$) which is probably converted to trivalent form *in vivo*, cannot produce permanent tolerance as intravenously injected $CrCl_3$ is bound mainly to serum proteins (Polák, unpublished results). Thus it is necessary to supplement the tolerogenic effect of $K_2Cr_2O_7$ bound to serum proteins in the trivalent form with the chromium hapten bound to specific skin carriers. This can only be provided by painting $K_2Cr_2O_7$ directly onto the skin.

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