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Studies on Contact Sensitivity to Chromium in the Guinea Pig. The Role of Valence in the Formation of the Antigenic Determinant

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Guinea pigs sensitized with either the trivalent chromium chloride or the hexavalent potassium dichromate are capable of reacting in vivo and in vitro to challenges with both chromium salts. This double reactivity is retained also after repeated restimulations with only 1 of these chromium compounds. From the failure to select lymphocytes directed specifically against a chromium determinant of a particular valence it is concluded that by sensitization with chromium salts of different valences a common determinant or closely related determinants are formed. It is suggested that this determinant is formed by chromium in the trivalent form.

Allergic contact eczema to chromium compounds, besides nickel eczema, is the most frequent occupational dermatosis [1,2]. Whereas the role of chromium salts in the induction and elicitation of contact dermatitis is undisputed, the exact form in which chromium participates in the formation of the antigenic determinant is still controversial.

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Abbreviation:

FCA: Freund's complete adjuvant

Chromium has 6 valence states but only tri- and hexavalent salts are sufficiently stable to act as haptens. Initially, clinical experience indicated that only hexavalent salts are capable of eliciting chromium eczema [3] but, later, positive results were obtained with trivalent compounds also [4]. Therefore, it has been concluded [5] that from the clinical point of view the chromium hypersensitivity is not directed against chromium compounds of a particular valence and, consequently, the more precise term is chromium allergy rather than chromate allergy. However, this term did not remain undisputed and it has been suggested that independent hypersensitivities exist against trivalent and against hexavalent chromium compounds [6].

An attempt to clarify, in animal experiments, the significance of chromium valence for the induction and elicitation of allergic eczema failed [7]. It has been shown that both the trivalent chromium chloride and the hexavalent potassium dichromate possess an equal sensitizing capacity in guinea pigs but the latter elicited reactions of a higher intensity than the former [7].

In the present paper we attempted to solve this problem by using the technique of positive or negative in vitro and in vivo selection of chromium-specific lymphocytes. The results of these experiments indicate that chromium-reactive lymphocytes are directed against common or very closely related determinants.

MATERIALS AND METHODS

Experimental Animals

Inbred strain 2 and partially inbred strain Rockefeller guinea pigs of either sex were used. They weighed about 350–400 g when sensitization was begun. The animals were bred at the Institute for Biochemical Research, Füllinsdorf, Switzerland. They were fed on pellet diet supplemented ad libitum with water containing vitamin C.

The sensitization was done in the following manner: 5 injections of 0.2 ml each of emulsion containing either 1 mg/ml of potassium dichromate (Merck, Darmstadt, FRG) in Freund's complete adjuvant (FCA) (DIFCO Laboratories, Detroit, Michigan) or 2 mg/ml of chromium chloride (Merck, Darmstadt, FRG) in FCA were given into the footpad and nape of the neck.

Animals were restimulated once a week by an intradermal injection of 25 μg either of potassium dichromate or chromium chloride in 0.1 ml 0.15 M NaCl solution into the skin of the right flank. Simultaneously 0.02 ml of either 0.5% potassium dichromate or chromium chloride solution in 1% Triton X-100 were applied epicutaneously on the skin of the left flank. The boosting was continued weekly until a positive reaction to the hapten was observed. The animals were then challenged epicutaneously with both haptens and the skin inflammation evaluated 24 hr later according to an arbitrary scale: red, swollen = 2, red, confluent = 1, red spots = 0.5.

Lymphocyte suspension was prepared from draining lymph nodes 14 days after sensitization. The cells were teased from the nodes with specially constructed forks and filtered through nylon stocking mesh and through 5-ml columns containing 1 ml nylon wool (Leuko Pak, Fenwal Lab., DIFCO Travenol Lab. Inc., Deerfield, Illinois). This suspension contained about 80% T cells, 10–15% B cells, and 5% macrophages.

Macrophages

Normal guinea pigs were injected with 20 ml light paraffin oil intraperitoneally (Drake Oil 6-VR White, Penna, Butler, Pennsylvania). The peritoneal exudate cells, containing about 70–80% macrophages, 10–15% lymphocytes, and 10–15% granulocytes, were washed out 4 days later with Hanks' balanced salt solution (GIBCO Biocult, Glasgow, Scotland), pooled and irradiated with 3300 r. The cells were then incubated 10 min at 37°C with either 3 mg/ml chromium chloride or 300 μg/ml potassium dichromate. After 2 washings the modified macrophages were counted and corresponding numbers (see below) were added to the culture.

Long-term Culture

Lymphocytes (2×10^7) were cultured in 10 ml RPMI 1640 medium (GIBCO Europe, Glasgow, Scotland) containing 50 μg/ml gentamycin (Schering Corporation, Kenilworth, New Jersey), 100 μg/ml streptomycin and 100 IU penicillin (GIBCO), 10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Missouri), 2 mM glutamin (GIBCO), 1 mM sodium pyruvate (GIBCO), 1 mM nonessential amino acids (GIBCO), and 5% heat-inactivated fetal calf serum (GIBCO) at 37°C in a humified atmosphere of 5% CO_2 and 95% air in tissue culture flasks (Falcon No. 3013, Oxnard, California). To the lymphocytes were added either 5×10^6 chromium^{III}- or chromium^{VI}-modified macrophages. Twice a week 4 ml of the culture medium was carefully replaced by fresh medium and on day 7, 5×10^6 haptenized macrophages were supplied in addition to the medium exchange. On day 14 the lymphocytes were harvested and 1×10^5 cells were cultured with either 1×10^5 chromium^{III}- or chromium^{VI}-modified or normal macrophages in 1 ml RPMI 1640 medium (GIBCO) containing 5% heat-inactivated guinea pig serum in culture tubes (12 × 75 mm, round-bottom plastic tubes, Falcon). Three days later the culture was pulsed for the last 18 hr with 1 μCi of [^{3}H]-thymidine (TRA 120, sp act 5 Ci/mM, Radiochemical Centre, Amersham, England) per tube and DNA synthesis was determined by counting in a liquid scintillation spectrometer using glass fiber filters (Whatman Inc., Clifton, New York).

Short-term Culture

Lymphocytes (5×10^5) were cultured with either 2×10^5 normal or chromium^{III}- or chromium^{VI}-modified macrophages in 1 ml RPMI 1640. Four days later they were pulsed with [^{3}H]-thymidine and counted as above.

RESULTS

In Vitro Selection of Chromium-Reactive Lymphocytes

Lymph node lymphocytes from guinea pigs primed with potassium dichromate in FCA exhibited a significant proliferative response upon stimulation with either chromium chloride-modified (Cr^{III}-Mø) or potassium dichromate-modified (Cr^{VI}-Mø) macrophages in vitro. This response was stronger upon stimulation with Cr^{III}-Mø than with Cr^{VI}-Mø (Table I). On the other hand, lymph node lymphocytes from chromium chloride in FCA-sensitized animals failed to show an enhanced in vitro DNA synthesis regardless of the valences of chromium salts used for haptenization of macrophages. However, it should be mentioned, that on some occasions lymphocytes from potassium dichromate-sensitized guinea pigs also failed to become stimulated by macrophages modified by chromium salts of either valence in vitro (data not shown).

In further experiments it has been shown that lymphocytes that responded to the first in vitro stimulation as well as lymphocytes that failed to do so exhibited a significant proliferative response when restimulated twice with chromium-modified macrophages during an expanded culture period (3 weeks). This increased [^{3}H]-thymidine incorporation could be elicited in lymphocytes from guinea pigs sensitized in vivo with chromium salts of either valence in FCA.

The restimulations with Cr^{III}-Mø induced a significantly higher proliferative response than restimulations with Cr^{VI}-Mø, and this effect was independent of whether the lymphocytes originated from chromium chloride- or potassium dichromate-primed guinea pigs. However, no significant difference in [^{3}H]-thymidine incorporation could be observed with respect to the valence of the haptenized macrophages used for the elicitation of the in vitro response, although in all experiments the Cr^{III}-Mø elicited a somehow stronger response.

Selection of in Vivo Response to Challenges with Chromium Salts of Different Valences

All guinea pigs sensitized with potassium dichromate in FCA and boosted several times epicutaneously and intradermally with the same hapten responded after 4–6 weeks to an epicutaneous challenge with this hexavalent chromium salt (Table II). At the same time only 7 of the 11 animals responded to a challenge with the trivalent chromium chloride. This inflam-

TABLE I. *Lack of in vitro selection of lymphocytes responding to chromium-modified macrophages of different valences*

Treatment in vitro		$CrCl_3$/FCA		$K_2Cr_2O_7$/FCA	
Restimulation in vitro	Challenge in vitro	cpm	S.I.	cpm	S.I.
none	Cr^{III}-Mø	1,244 ± 335	1.5–2.5	10,596 ± 1,779	3.3–38.6
	Cr^{VI}-Mø	1,419 ± 574	1.4–2.4	4,749 ± 836	3.3–13.0
Cr^{III}-Mø	Cr^{III}-Mø	88,996 ± 27,313	6.5–15.9	41,060 ± 3,880	6.6–10.8
	Cr^{VI}-Mø	48,999 ± 21,495	2.0–7.5	28,162 ± 13,698	2.1–11.8
Cr^{VI}-Mø	Cr^{III}-Mø	8,589 ± 2,832	4.4–5.1	8,256 ± 1,629	3.2–12.3
	Cr^{VI}-Mø	6,649 ± 4,462	1.3–4.5	5,807 ± 3,359	3.0–3.2

Lymph node lymphocytes from guinea pigs sensitized with either chromium^{III} chloride ($CrCl_3$) or potassium dichromate ($K_2Cr_2O_7$) in FCA were assayed for in vitro DNA synthesis upon stimulation and restimulations with chromium^{III}- or chromium^{VI}-modified macrophages (Cr^{III}-Mø; CR^{VI}-Mø). Results are expressed as mean increments in cpm and as minimal and maximal stimulation index (S.I.).

TABLE II. *Selection of in vivo response to challenges with chromium salts of different valences*

Sensitization in vivo	Restimulation e.c. + i.d.	Challenge e.c.	Number of animals: Positive/Total	Animals reacting positively to both haptens: Same intensity to both haptens	Stronger reaction to challenge with the sensitizing hapten
$K_2Cr_2O_7$ in FCA	$K_2Cr_2O_7$	$K_2Cr_2O_7$	11/11	4/7 (57%)	3/7 (43%)
		$CrCl_3$	7/11		
$CrCl_3$ in FCA	$CrCl_3$	$CrCl_3$	7/10	3/3 (100%)	0
		$K_2Cr_2O_7$	3/10		

Guinea pigs were sensitized with $CrCl_3$ or $K_2Cr_2O_7$ in FCA and boosted weekly with the same hapten until positive reaction occurred (maximum 6 weeks). Then the animals were challenged with both haptens simultaneously and the reactions evaluated 24 hr later. Abbreviations: e.c. = epicutaneously; i.d. = intradermally.

matory skin reaction was of the same intensity to both chromium compounds in 4 and of a weaker intensity to chromium chloride than to potassium dichromate in 3 of the 7 guinea pigs responding to both haptens. Four guinea pigs responded only to potassium dichromate.

Essentially, the same results were observed in guinea pigs sensitized and boosted with the trivalent chromium chloride. From the 7 animals that responded to an epicutaneous challenge with chromium chloride, 3 responded also to potassium dichromate. In the guinea pigs that responded to both haptens, no difference in the intensity of these reactions could be observed.

Chromium-modified macrophages used for in vivo sensitization gave erratic results regardless of the valence of chromium salts used for haptenization (data not shown).

DISCUSSION

The aim of our experiments was to determine which is the relevant determinant in chromium contact sensitivity with respect to the chromium valence. By evaluating the clinical and experimental data [7] two possibilities became apparent: either one common determinant is formed by sensitization with both trivalent or hexavalent chromium compounds, the latter probably after conversion into its trivalent form [8], or the determinant formed by sensitization with hexavalent chromium salts differs from the one formed by sensitization with the trivalent salts.

The first possibility was indicated by the fact that lymphocytes obtained from guinea pigs sensitized to a chromium compound of a certain valence respond in vitro to stimulations with chromium compounds of both valences. However, it appeared to be of importance—to exclude this possibility—that by application of the hapten in vivo 2 or more determinants consisting of chromium of different valences are formed. In that case, lymphocytes from these animals would also respond both in vivo and in vitro to chromium salts of both valences.

In order to clarify this problem the technique of positive in vitro selection was used. It has been shown [9] that lymphocytes from guinea pigs sensitized to 2 different antigens and repeatedly in vitro stimulated with 1 of them lose their capacity to respond to the other one. From these results it is evident that in long-term culture only lymphocytes with specificity to the hapten used for restimulations survive. Consequently if the determinants formed by chromium chloride and potassium dichromate were different, responsiveness to only the one used for restimulations would be maintained.

This was, however, not the case in our experiments. Lymph node lymphocytes from animals sensitized with potassium dichromate did not lose their ability to respond to chromium chloride-modified macrophages (Cr^{III}-Mø) even after repeated in vitro restimulations with potassium dichromate-modified macrophages (Cr^{VI}-Mø). On the contrary, the proliferative response to Cr^{III}-Mø was always of a slightly higher degree than the response to Cr^{VI}-Mø regardless of the valence of the chromium compounds used for in vivo priming and for in vitro restimulations. The capacity to respond in vitro to both Cr^{III}-Mø and Cr^{VI}-Mø was retained in cultures for up to 6 weeks regardless of whether tri- or hexavalent chromium compounds were used for sensitization and restimulations. The proliferative response to both Cr^{III}-Mø (161,863 cpm) and Cr^{VI}-Mø (182,888 cpm) was only slightly lower than the level of incorporation reached by mitogenic stimulation with PHA (236,416 cpm), indicating a substantial enrichment of specific antigen-reactive cells.

These results could be considered as evidence for the existence of 1 common determinant formed by contact with chromium compounds of either valence. It is, however, conceivable that by in vivo or in vitro contact with a hapten, several determinants differing in their protein component(s) are formed [10]. One may speculate that during sensitization with chromium salts of different valences several common determinants are formed, as well as some additional determinants specific for the chromium salt of a particular valence. The existence of common determinants might be the reason for the maintenance of the simultaneous response to chromium salts of both valences, whereas the additional specific ones may be responsible for the differences in intensity of the immune response to a particular hapten.

Another unsolved problem concerning the determinant(s) against which chromium hypersensitivity is directed is the valence of chromium contained in the antigenic complex. From the literature it is known that only trivalent chromium compounds are capable of forming covalent bonds with proteins [11], which is the precondition for immunogenicity [12]. On the other hand, it has been demonstrated that hexavalent chromium is converted into the trivalent form by sulfhydryl group-containing amino acids in the skin [8]. Since these amino acids are also present in the medium used for lymphocyte cultures, the conversion may also occur in vitro.

The idea that the chromium reactive-lymphocytes are directed against the trivalent form is further supported by our results, demonstrating the better in vitro stimulatory capacity of Cr^{III}-Mø than of Cr^{VI}-Mø. This hypothesis is further favored by our previous reports showing that lymphocytes from chromium-sensitive guinea pigs are capable of producing macrophage migration inhibition and skin reactive factors only upon stimulation with the trivalent chromium chloride but not with the hexavalent potassium dichromate [7]. In the direct macrophage migration inhibition assay, both chromium salts were equally active.

The higher amounts of chromium chloride necessary for a successful lymphocyte stimulation in all these tests could be explained by the binding of this protein-active compound to some immunologically irrelevant proteins. On the other hand, the lower amount of potassium dichromate sufficient for efficient haptenization of macrophages and for inhibition of their in vitro migration could be understood by assuming that the relevant protein binding as well as the antigen processing occurs inside these cells [13]. It is known [7] that the hexavalent chromium penetrates cell membranes much more easily than the trivalent chromium. However, hexavalent chromium had to be converted into its trivalent form inside the macrophages in

order to be able to bind proteins and to be presented to T cells. All of these considerations lead to the conclusion that the antigenic structure recognized by specific lymphocytes contains chromium in its trivalent form since a conversion in the opposite direction, i.e., from hexavalent to trivalent, was never demonstrated.

The sometimes better in vivo sensitizing capacity of hexavalent chromium salts in comparison to the trivalent ones may be the consequence of better penetration into the skin, which facilitates its processing and presentation by Langerhans cells [14].

Our in vivo results showing a certain degree of selectivity upon repeated restimulation with the particular hapten could be interpreted by an existence of 2 entirely different determinants, one resulting from the sensitization and restimulations with potassium dichromate and the other from the sensitization and restimulations with chromium chloride. However, a closer analysis of the results seems to refute this interpretation. In all chromium chloride- and in about 50% of potassium dichromate-sensitized guinea pigs that reacted to an epicutaneous challenge with both haptens, the intensity of these reactions was not different, indicating that at least in these animals no selection for the respective determinant took place. In the other guinea pigs reacting only to the hapten used for sensitization and restimulations, some nonimmunologic factors such as penetration capacity, conversion of hexavalent to trivalent forms, and protein binding activity may be responsible for the negative or weaker reactions to the chromium salt not used for sensitization and restimulations. This view is supported by the lack of selection in in vitro experiments, where these factors are of lower importance or excluded.

The other possibility, which has been mentioned above, is that besides several common determinants some additional determinants which are formed in vivo by application of chromium chloride may differ from those formed by application of potassium dichromate. This may be due to the fact that in vivo chromium chloride is bound to the proteins on the surface of macrophages, whereas potassium dichromate forms the relevant conjugates inside the cells after penetrating the membrane and conversion into its trivalent form. This difference in the antigenic formation site is less pronounced in in vitro experiments probably because of the less complicated mechanisms of haptenization of macrophages in vitro as compared to hapten processing and presentation in vivo.

Thus, the results of our in vivo experiments do not contradict the possibility that chromium sensitivity is mainly directed against 1 common determinant and that this determinant contains chromium in its trivalent form.

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