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Antigen Specific Human T Cell Lines Specific for Cobalt Chloride

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T lymphocyte lines specific for cobalt ($CoCl_2$) were established from one cobalt and nickel sensitive donor. The lines were generated by stimulating peripheral blood mononuclear cells (PBL) with $CoCl_2$ for 7 days. The activated blasts were maintained in interleukin 2 containing media and every 7–10 day restimulated with antigen ($CoCl_2$) and fresh irradiated PBL. The antigen specificity of the lines was attested by their capacity to proliferate and release of IL 2 under restimulation by $CoCl_2$ and not by an antigen like $NiSO_4$, towards which the donor was also sensitized. The cell lines were of the helper phenotype, T3/T4 positive subset, as determined by monoclonal antibodies. Analysis of the HLA class II restriction by using allogeneic PBL as antigen presenting cells and the capacity of anti HLA-D antibodies to effectively inhibit the specific response indicated that the antigen specific T cell response to contact allergens like cobalt chloride is restricted to HLA class II antigens. *Key words: Contact sensitivity; HLA restriction.* (Received October 23, 1985.)

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Contact sensitivity is despite its common occurrence a condition with an imperfectly known pathogenesis. The classical allergic contact eczema is considered to be a delayed type hypersensitivty (DTH) reaction to an antigen consisting of a small molecular weight hapten and autologous skin proteins. In consistence with other DTH reactions, like the tuberculin reaction, the crucial role of the T lymphocytes in the immune response is well established (1). DTH reactions are initiated by events involving T cell recognition of the external antigen predominantly in association with antigens of the major histocompatibility complex (MHC) class II, in man the HLA-D antigens, on the antigen presenting cell (APC) (2–4). In skin, the Langerhans cell is the main APC, while APC in peripheral blood are monocytes and dendritic cells. Detailed analysis of the elicitating mechanisms as well as the immune regulation of contact sensitivity do require a supply of lymphocytes highly enriched for T cells specific for the sensitizing hapten.

In man, antigen specific T cell lines and clones have been isolated for various antigens including purified protein derivative of tuberclin (PPD), tetanus toxoid, Chlamydia trachomatis and ragweed antigen E (5–9).

Generating antigen specific T cell lines is greatly facilitated by the discovery and characterization of interleukin 2 (IL 2 or T cell growth factor) a lymphokine produced and released by antigen specific T cells while stimulated with the proper antigen (10, 11).

The present study describes the generation and characteristic features of human T cell lines specific for cobalt chloride ($CoCl_2$) established from a person allergic to cobalt as well as to nickel.

MATERIALS AND METHODS

Metal salt solutions

$CoCl_2 \times 6\ H_2O$ and $NiSO_4 \times 6\ H_2O$ (Merck, Germany) were prepared as 1% stock solutions in distilled water. The stock solutions were diluted in medium RPMI 1640 (Gibco, Paisley, Scotland)

containing penicillin, streptomycin and 2 mM L-glutamine to the final concentrations in the cultures of 0.3–10 μg/ml for $CoCl_2$ and 3.1–25 μg/ml for $NiSO_4$.

Preparation of IL 2 containing medium

Peripheral blood mononuclear cells (PBL) from healthy blood donors were cocultured at 2.5×10^6/ml for 2 hours with 5 μg/ml phytohemagglutinin (PHA) (Sigma) and 5 ng/ml phorbol myristic acetate (Sigma) in medium RPMI 1640 supplemented with 1% pooled human AB-serum. Subsequently the cells were washed extensively and resuspended in medium supplemented with 1% pooled human AB-serum. After 40 hours culture the supernatant was harvestered filtered through 0.45 μm filter and stored at −20°C. The IL 2 containing supernatant was usually used at a final concentration of 15–30% in culture.

Generation of cobalt specific T cell lines

For the experiments we selected one otherwise healthy adult volunteer (HLA-DR 2/3) that has a multiple metal allegy. She is extremely sensitive to cobalt and also but less to nickel. Peripheral blood mononuclear cells (PBL) were cultured at 1×10^6/ml in medium RPMI 1640 supplemented with 10% pooled human AB-serum or 10% autologous plasma and $CoCl_2$ 1 μg/ml in 50 ml tissue culture flasks (Nunc, Denmark) at 37°C in humidified air containing 5% CO_2. Seven days later the culture was harvested and the blasts were separated by Ficoll-Paque gradient (Pharmacia, Fine Chemicals, Uppsala, Sweden). The activated blasts were then restimulated with 1 μg/ml $CoCl_2$ and fresh irradiated autologous PBL as antigen presenting cells. After 3–4 days the cobalt activated blasts were separated by Ficoll-Paque gradient and replaced in culture at 1×10^5/ml in RPMI 1640 medium supplemented with IL 2. Every 2–4 days the cells were replaced in new IL 2 containing medium. The cell concentration was always kept below 5×10^5/ml. At 7–10 days interval the cell lines were restimulated with $CoCl_2$ 1 μg/ml and fresh irradiated autologous PBL.

Test for antigen specific proliferative response

The cell lines were tested for their response against the antigen in a 3-day assay. 2×10^4 Co-activated blasts were cocultured with 2×10^5 fresh autologous irradiated (2500 R) PBL in the presence or absence of antigen in flat bottomed microtitre plates (Nunc, Denmark) in culture medium containing 10% pooled human AB-serum or 10% autologous plasma in a total volume of 200 μl. Twenty hours before the cultures were harvested 1 μCi ^{3}H-thymidine was added to each well. The cells were then harvested on fibre glass filters using a semiautomatic multiple harvester (Skatron, Lierbyen, Norway). The dried filters were assayed in a liquid scintillation counter (LKB, Wallac, 1218 Rackbeta, Finland). Results were expressed as mean counts per minute (cpm).

Induction and assay of IL 2

Cell lines were taken from the in vitro resting state, that means the cells were growing in IL 2 containing media and had not been exposed to antigen and antigen presenting cells for at least 7 days.

Dead cells and debris were removed by centrifugation over Ficoll-Paque gradients (Pharmacia, Uppsala). The cells were washed twice with PBS-D (Gibco, Scotland) and cultured in flat bottomed microtitre plates with antigen and autologous irradiated PBL at the same conditions as previously described for the proliferation assay. The culture supernatants were removed after 24 hours and the possible IL 2 activity was tested on the IL 2 dependent mouse T cell line CTLL (12). Presence of IL 2 in the supernatants of the antigen activated T cell lines was assessed by measurements of the incorporation of ^{3}H-thymidine by CTLL cells in the presence of 6.25–50% (v/v) supernatant in 24 hour proliferation assay.

Cell surface marker analysis

Phenotypic analysis was performed by a micro-panning method as described in detail (13). Briefly, microtitre plates were coated with $F(ab)_2$ fragments 100 μg/ml of affinity purified goat anti mouse IgG (Cappel, Cochranville, PA) over night at 4°C, after washing the plates with PBS-D monoclonal mouse anti human T cell antibodies (see below) were added. After 1–2 hours at 37°C the plates were again washed and the cells to be tested for adherence to the monoclonal antibodies were added. The plates were incubated for 1–2 hours at 4°C and then washed in PBS-D. The cell binding to the monoclonal antibodies was monitored using an inverted microscope.

Surface markers were detected by the following monoclonal antibodies: OKT 3 (Ortho Pharmaceuticals, Raritan, NJ) directed towards the T3 complex on mature peripheral T lymphocytes; OKT 4 (Ortho) and Leu 3a (Becton Dickinson, Mountain View, CA) detecting the T4 molecule on predominantly T 'helper/inducer' cells; with class II MHC specificity (14, 15); OKT 8 (Ortho) and Leu 2a (Becton Dickinson) directed towards the T8 molecule on the subset with predominant T 'suppressor/cytotoxic' cells, specific for class I MHC (16, 17).

Inhibition of cell proliferation by anti HLA antibodies

The effect of anti HLA antibodies in the proliferation assay was tested by using rabbit heteroantisera directed against HLA antigens in man (provided by Dr L. Klareskog. Department of Medical and Physiological Chemistry, Biomedical Centre, University of Uppsala).

The antiserum against the products from the HLA-D gene locus, the MHC class II antigens, was an IgG fraction of the rabbit antiserum prepared by purification on protein A-coupled Sepharose affinity chromatography columns (18), followed by immunosorbent chromatography on Sepharose coupled HLA-D antigens isolated from chronic lymphatic leukaemia cells (19). As antiserum against the MHC class I antigens we used antiserum raised against β_2-microglobulin.

The HLA-D antiserum reacts with antigenic determinants shared between the different allelic forms of HLA-D antigens (class II antigens). The β_2-microglobulin antiserum does recognize the light invariable chain of the HLA-A, -B, -C antigens (class I antigens). The effect on the cell proliferation of the anti HLA antibodies was tested by adding the antisera in different dilutions from start of the cultures. Normal rabbit serum was used as control serum.

RESULTS

Generation of cobalt specific cell lines

PBL from a panel of donors including some with a contact sensitivity to nickel and/or cobalt and others without any known contact allergy were tested for the ability to proliferate in the presence of $NiSO_4$ and $COCl_2$ in a primary proliferative assay (data not shown).

One individual, donor CK, that showed a reproducible and very strong primary proliferative in vitro response especially to $CoCl_2$ compared to the other cobalt sensitive persons, was selected for further studies. Several cobalt specific T cell lines were established from peripheral blood mononuclear cells from this donor by repetitive restimulations with antigen combined with periods in between where the cell lines were kept in IL 2 supplemented media.

At different time intervals in culture the cell lines were tested for the proliferative response against $CoCl_2$ and autologous irradiated PBL as antigen presenting cells. As specificity control the cell lines were simultaneously stimulated with $NiSO_4$ and autologous PBL. A shown in Table I the cell lines retained their antigen specific activity over the entire test period.

Besides measuring the ^{3}H-thymidine uptake in the proliferating cells, when triggered with the proper antigen, we also measured the capability of the cells to release IL 2 when stimulated with relevant antigen. Supernatants were assayed for IL 2 activity, 24 hours after the onset of cultures while the ^{3}H-thymidine uptake in the proliferating cells was

Table I. *Antigen specific proliferation (SI) of T cell lines, at different times in culture*

SI = mean cpm stimulated cultures/mean cpm unstimulated cultures. ND = not determined. Cobalt activated blasts were cultured with or without antigen and irradiated autologous PBL. Data shown is for day 3 ^{3}H-thymidine incorporation of triplicate cultures

Cell line	Days in culture	Background (cpm)	Antigen added	
			$CoCl_2$	$NiSO_4$
10/83	20	2 881	5.2	ND
6/84	25	5 868	7.3	0.8
9/83	41	6 612	3.5	0.6
	49	16 765	6.6	0.5
2/83	36	691	109.0	1.8
	70	3 139	26.5	1.4

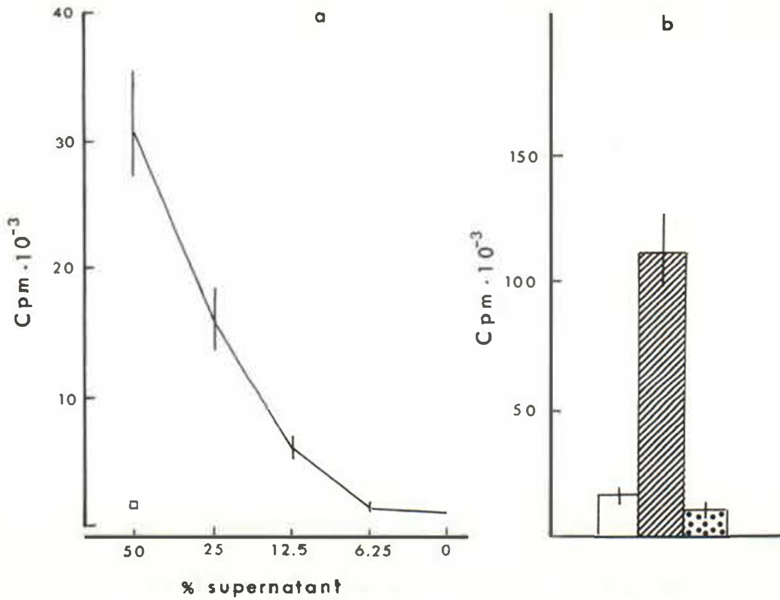

Fig. 1. Antigen triggered release of IL 2 from the cobalt specific T cell line. (*a*) Proliferation (cpm±SD, triplicate cultures) of IL 2 dependent CTLL cells, 4×10^3 cells/well in the presence of supernatant from antigen stimulated cobalt specific T cell line. ——, supernatant from $CoCl_2$ (1 μg/ml) stimulated cultures; □, 50% supernatant from $NiSO_4$ (6.25 μg/ml) stimulated cultures. The supernatant is harvested 24 h after the onset of culture. (*b*) Proliferation (cpm±SD, triplicate cultures) of antigen stimulated cobalt specific T cell line. □, T cells (10^4)+autologous PBL (10^5)/well in medium; ▩, T cells + PBL + 1 μg/ml $CoCl_2$; ▩, T cells + PBL + 6.25 μg/ml $NiSO_4$. The proliferation is assayed 48 h after the onset of culture.

tested after 48 hours in culture. From the result presented in Fig. 1 it is clear that the cobalt specific cell lines are capable of producing IL 2 and to proliferate when stimulated with $CoCl_2$ but not with $NiSO_4$.

Surface markers analysis of cobalt specific cell lines

The T cells were characterized with regard to ability to bind monoclonal antibodies directed to different subpopulations of T lymphocytes. The panning technique that was used will effectively immobilize the cells provided the cell surface antigen is present on the cells. Three different cobalt specific T cell lines reacted identically. They all reacted with the OKT 3 and OKT 4 or Leu 3a antibodies. No cell line reacted with the OKT 8 or Leu 2a antibodies. In accordance with recent data (14–17) one should thus consider our T3/T4+ T cells to be HLA class II restricted T cells presumably of 'T-helper cell' type.

HLA class II restriction of the T cell response

HLA class II antigens are products coded for by genes within the D region of the human histocompatibility complx. There are at least three well defined HLA class II products called HLA-DR, HLA-DQ and HLA-DP. HLA-DR is the most studied and well defined locus (20, 21).

In order to study the possible HLA class I or II restriction in the antigen specific proliferation of the T cell lines we first tested allogeneic PBLs from donors sharing at least one DR haplotype with the donor of the cobalt specific cell lines as antigen presenting cells. The results in Table II show, however, that only the autologous situation gave a

Table II. *Antigen specific proliferation of T cell lines in presence of autologous and allogeneic PBL*

SI = mean cpm stimulated cultures/mean cpm unstimulated cultures. Cobalt activated CK-blasts were cultured with or without $CoCl_2$ 1 µg/ml and irradiated autologous and allogeneic PBL with known HLA-DR specificity. Data shown is for day 3 ^{3}H-thymidine incorporation of triplicate cultures

Donor of PBL	HLA-DR	SI
Experiment 1		
CK (autologous)	2.3	26.5
LK	2.4	0.9
AL	3.3	1.5
Experiment 2		
CK (autologous)	2.3	5.2
BA	3.–	1.7

good proliferative response and with the available donor panel no safe HLA-restriction was noted. However, antibodies directed against the nonpolymorphic region of HLA-D antigens brought about a very strong inhibition when included in the proliferation assay, whereas anti class I HLA antibodies failed to suppress (Table III).

Table III shows that anti HLA-D antiserum up to a dilution of 1:1350 will abolish the proliferative response more than 75%. On the other hand anti β_2-microglobulin antiserum that recognizes the invariable small chain of the HLA-A, -B, -C antigens if anything cause a weak amplification of the proliferative response.

Taken together the above experiments indicate that the antigen triggered proliferation of the Co-specific T cell lines is HLA-D but necessarily not HLA-DR restricted.

DISCUSSION

In the present study we demonstrate the generation and characterization of antigen specific human T cell lines highly specific for cobalt chloride.

The cell lines were obtained from a single donor with a contact allergy to both nickel and cobalt. To establish the cell lines we stimulated PBL with a combination of $CoCl_2$ and

Table III. *Inhibition of antigen specific proliferation with anti HLA antiserum*

Cobalt activated blasts were stimulated with antigen and irradiated autologous PBL in the presence of various dilutions of rabbit anti HLA-D antiserum and anti β_2-microglobulin antiserum. The data presented are relative responses compared to the value in the presence of normal rabbit serum

Antiserum dilution	Relative response %	
	anti HLA-D	anti β_2-microglobulin
1:50	18.4	62.3
1:150	20.0	50.7
1:450	32.2	137.3
1:1 350	25.0	113.4
1:4 050	74.6	110.4

autologous irradiated PBL and interleukin 2 (IL 2) for more than 2 months. During this period the cells retained antigen specificity as determined by proliferation and/or release of IL 2 when stimulated by the appropriate antigen. The antigen specific cell proliferation and secretion of IL 2 by the cell lines occurred under similar conditions. Both required the addition of autologous PBLs as antigen presenting cells (APC) and the proper antigen. Attempts to induce proliferation and release of IL 2 by stimulating the cobalt reactive T cell lines with a closely related hapten like nickel sulphate towards which the donor is also sensitized always failed. Besides confirming the antigen specificity of the T cell lines the failure shows convincingly that cobalt and nickel do not behave as cross reacting haptens. The existence of a cross-sensitivity between cobalt and nickel among allergic patients has been discussed, but has been rejected by among others Pirilä & Förström (22).

Although not properly cloned the cell lines thus behave in specificity test in the same way. In agreement with this, three different cobalt specific cell lines all had the same surface antigen phenotype and belonged to the T3/T4 positive subset as determined by monoclonal antibodies. It is well established that induction of T cell responses involves recognition of the external antigen in association with the antigens of the major histocompatibility complex, in man the HLA antigens (3, 4). Furthermore it has been shown that the proliferative T cell response to protein antigens like PPD and tetanus toxoid normally are restricted to the HLA class II antigens (5–7). The class II or HLA-D antigens comprise at least three well defined and disparate groups of molecules of the types denoted DR, DQ and DP (20, 21).

Our results strongly suggest that the T cell response to metal compounds such as cobalt chloride is restricted to HLA-D antigens. The phenotype of the T cells is thus suggestive of HLA-D restriction (14, 15). Antibodies specific to the HLA-D antigens also effectively inhibited the specific response when the T cell lines are stimulated with the proper antigen and autologous PBL whereas anti class I MHC antibodies failed. However, we have so far been unable to induce any significant cell proliferation using allogeneic PBL instead of autologous PBL as antigen presenting cells even when the T cell lines and the donor of the allogeneic PBL did share one HLA-DR determinant. It has recently been shown, however, that other defined HLA-D antigens such as DP and DQ can also act as restriction elements for the antigen specific T cell proliferation as well (23–25). Consequently it is likely that the antigen-specific T cell response to contact allergens like cobalt chloride is restricted to HLA-D antigens.

In further analysing the elicitating mechanisms and the immunoregulation of contact sensitivity more extensively, established antigen specific T cell lines and especially clones should prove extremely valuable because of the homogeneity of the cell population. We will accordingly use such cells to further explore the molecular nature behind the restricted cobalt immunity demonstrable in specifically allergic individuals.

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