Brief Definitive Report

ANTIGEN RECOGNITION BY HUMAN T CELL RECEPTOR γ–POSITIVE LYMPHOCYTES

Specific Lysis of Allogeneic Cells after Activation in Mixed Lymphocyte Culture

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The surface molecules that serve as receptor for antigen/MHC recognition are composed of a disulphide α/β heterodimer that is noncovalently associated with CD3, a multipolypeptide cell membrane complex (1, 2). In addition, a minor cell subset has recently been identified that expressed CD3-associated structures that represent the molecular product of the TCR-γ gene (3). TCR-γ^+ cells differ from typical TCR-α/β^+ T cells for both phenotypic and functional properties. Thus, they lack both CD4 and CD8 differentiation antigens, which are known to identify the two major peripheral T cell subpopulations; they also lack CD28 (4), a surface molecule involved in the activation of typical TCR-α/β cells, but express CD11 and LAK-1, surface markers expressed by human NK cells (5). In addition, virtually all TCR-γ^+ cells give rise to cytolytic clonal progenies with lytic activity against a variety of tumor target cells (6). Previous studies have shown that stimuli acting on the TCR/CD3 molecular complex induce TCR-γ^+ cells to express their functional program (including lymphokine production and activation of the lytic machinery) in a manner that is indistinguishable from conventional TCR-α/β^+ cells (7). However, no information is so far available on the natural ligand responsible for human TCR-γ^+ cell activation. The fact that the TCR-γ chain, similarly to α and β chains, is formed by multiple rearranging genes that code for variable regions, supports the idea that antigen(s) may be the natural ligand(s) also for TCR-γ^+ cells. In an attempt to verify this hypothesis, we analyzed the ability of TCR-γ^+ cells to recognize alloantigens. We show that purified CD4$^-$8$^-$ TCR-γ^+ cells can proliferate in response to allogeneic cells in MLC; the resulting MLC populations display a strong cytolytic

activity against specific target cells (but not against unrelated or autologous targets). In addition, the expression of CD3-associated molecules different from TCR-α and -β chains, together with the detection of γ mRNA and the lack of α and β mRNAs, suggests that CD3$^+$4$^-$8$^-$ MLC-activated populations indeed express the TCR-γ gene product. Finally, several TCR-γ^+ clones have been obtained from different individuals, which display a restricted cytolytic pattern.

Materials and Methods

Isolation of CD3$^+$4$^-$8$^-$ Lymphocytes, MLC, Evaluation of Cytolytic Activity and Cell Cloning. Peripheral blood lymphocytes from normal volunteers were isolated by Ficoll-Hypaque (F-H) gradients and separated into E rosetting–positive (E$^+$) and –negative (E$^-$) populations. E-rosetting cells were then incubated with a mixture of anti-CD4 and anti-CD8 mAbs followed by treatment with rabbit complement for 1 h at 37°C. Viable cells were isolated by F-H gradients and cultured in microwells (5 × 10^4 cells/well) in the presence of autologous or allogeneic irradiated feeder cells; after 7 d the cultures were supplemented with 100 U/ml of rIL-2 (Cetus Corp., Emeryville, CA). The MLC-derived cells were cloned under limiting diluition conditions in the presence of allogeneic irradiated feeder cells and 100 U/ml of rIL-2. The mAbs used in these experiments were the following: Leu-4 (anti-CD3) (Becton Dickinson & Co., Basel, Switzerland), HP26 (anti-CD4), B9.4 (anti-CD8), and MAR 206 (anti-CD2) (prepared in our laboratory; reference 7); WT31 (directed to a framework determinant of the TCR-α/β) (Sambio, Uden, The Netherlands).

The degree of cell proliferation in MLC was evaluated after 10 d of culture as the arithmetic mean of the number of cells recovered from six wells of each experimental group, or by the uptake of [^{3}H]thymidine, added 18 h before harvesting. The cytolytic activity was tested in a 4-h ^{51}Cr-release assay, in which varying numbers of effector cells were tested against one or another of the following target cells: NK-sensitive K562 cells, fresh uncultured melanoma cells derived from a malignant ascites, PHA-blasts derived from the same source of lymphocytes used as stimulating cells in MLC (or unrelated lymphocytes, as control). PHA blasts were obtained by culturing PBL for 4 d with 0.5% PHA (vol/vol) in the presence of rIL-2 (100 U/ml). In all instances, target cells were used at 5 × 10^3/well, for a final effector/target ratio ranging between 25:1 and 1.5:1. Percent specific lysis was determined as previously described (8).

Immunoprecipitation. Cells (5 × 10^6) were radioiodinated by the lactoperoxidase technique, lysed under conditions which preserve the CD3-TCR complex (in a buffer containing 1% digitonin) and immunoprecipitated as described (5, 7). After immunoprecipitation, samples were examined by SDS-PAGE under nonreducing or reducing conditions using 5% 2-ME, which cleaves disulfide bonds between protein subunits.

Northern Blot Analysis. Total cellular RNA was extracted from frozen cell pellets by the guanidinium thiocyanate method and purified by centrifugation through a CsCl gradient. RNA was size fractionated by electrophoresis in a 1.5% agarose gel containing formaldehyde in MOPS buffer and blotted into nitrocellulose buffer. Filter hybridization to the ^{32}P-labeled DNA probes and washings were performed as previously described (5). The cDNA probes for TCR-α and β chains were kindly provided by Dr. Tak Mak (9), whereas, for TCR-γ, we used a genomic probe containing the 3′ half of the first exon of the Cγ1 gene (a kind gift of Dr. T. Rabbitts, Cambridge, U. K.) (10).

Results and Discussion

Enriched CD3$^+$4$^-$8$^-$ WT31$^-$ cells were derived from peripheral E-rosetting cells by treatment with anti-CD4 plus anti-CD8 mAbs and complement. The resulting populations (2–10% of the starting cells) were CD4$^-$ and CD8$^-$, >99% were CD2$^+$ and contained variable proportions of CD3$^+$ cells (40–90%). <2% of treated cells were reactive with the WT31 mAb. Further purification based

on the positive selection of CD3$^+$ cells was not applied, since such selection would result in antibody-induced CD3/TCR modulation which could interfere with alloantigen recognition. Enriched CD3$^+$CD4$^-$8$^-$ cells were cultured in microwells with allogeneic or autologous irradiated mononuclear cells. IL-2 was added 7 d after initiation of the cultures and proliferation was evaluated 3 d later. Cell proliferation was evaluated either as [^{3}H]TdR uptake or as number of recovered cells/well. Specific cell proliferation in response to allogeneic cells (up to eightfold increase of the initial number of responding cells cultured) was detected in three of five individuals tested. In addition, most MLC-derived CD3$^+$ cells were also stained by an anti-TCR-γ mAb recently derived in our laboratory (manuscript in preparation). As indicated by surface marker analysis, the resulting MLC populations contained <1% WT31$^+$ or CD4$^+$ or CD8$^+$ cells. As shown in Fig. 1, these MLC responsive cells lysed ^{51}Cr-labeled PHA-induced blasts derived from the allogeneic stimulating cells, but not autologous or unrelated blasts (A). In contrast, the same population cultured with autologous irradiated cells did not develop any cytolytic activity, neither against autologous nor against allogeneic target cells (B). The cytolytic pattern of MLC-stimulated CD3$^+$4$^-$8$^-$WT31$^-$ cells in both allogeneic and autologous MLC was comparable to that of MLC-stimulated TCR-α/β^+ cell populations derived from the same donor (Fig. 1, C and D). To determine the nature of CD3-associated molecules expressed by alloreactive CD3$^+$4$^-$8$^-$WT31$^-$ cells, we performed surface labeling experiments, followed by lysis under conditions that preserve the CD3-TCR complex association, and immunoprecipitation with anti-CD3 mAbs. These experiments showed that, in two individuals analyzed (Fig. 2, panel 1), CD3-associated molecules consisted of two bands of 45 and 43 kD, respectively
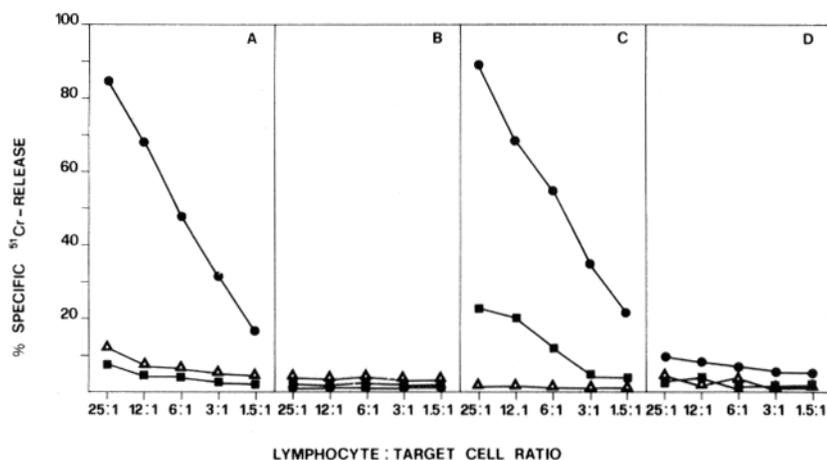

FIGURE 1.   MLC-activated CD3$^+$4$^-$8$^-$WT31$^-$ cells specifically lyse allogeneic target cells. Cells were stimulated in MLC as described in Materials and Methods and tested for cytolytic activity against different ^{51}Cr-labeled PHA-induced target cells. (A) CD3$^+$4$^-$8$^-$WT31$^-$ cells cultured with the allogeneic cells a lysed only target cells a, but not allogeneic target cells b or autologous cells. (B) The same cell population, after culture in the presence of autologous lymphocytes, was unable to lyse both autologous or allogeneic target cells. (C and D) Effector cells were represented by CD3$^+$WT31$^+$ cells derived from the same donor and activated either against allogeneic cells a or against autologous lymphocytes. Allogeneic target cells a, ●; allogeneic target cells b, ■; autologous target cells, △.

(under reducing conditions). The same two chains were immunoprecipitated by anti-γ peptide antisera (1–6) as well as by a recently derived anti-TCR-γ mAb (data not shown). Similar CD3-associated molecules were previously detected in immunoprecipitates from $CD3^+4^-8^-WT31^-$ clones or populations derived from normal peripheral blood (5, 7). We further investigated whether mRNAs for different TCR chains could be detected in polyclonal $CD3^+WT31^-$ alloreactive cell populations. Northern blot analysis (Fig. 2, panel 2) clearly demonstrated the presence of mature mRNA for the γ chain, while the mRNAs for the α and β chains were absent. All together, these data support the notion that the γ gene molecular product and not α or β chains were associated to CD3 molecules in our $CD3^+4^-8^-WT31^-$ alloreactive populations. We can therefore conclude that cells expressing TCR γ can specifically recognize and lyse allogeneic cells.

To further document the ability of TCR-γ^+ cells to specifically recognize (allo) antigens, we derived several clones from MLC-stimulated $CD3^+4^-8^-WT31^-$ populations. In all instances the clones analyzed expressed the original $CD2^+3^+4^-8^-WT31^-$ phenotype (not shown). The cytolytic pattern of seven representative clones is shown in Table I. It is evident that the clones maintained the same cytolytic capability of the original MLC population. Thus, only specific allogeneic target cells, but not autologous or unrelated allogeneic ones, were lysed. Moreover, the specific cytolytic activity against allogeneic cells was not necessarily linked to the expression of MHC-unrestricted cytotoxicity against NK-sensitive or NK-resistant tumor target cells. In fact, clone C11 lysed specific allogeneic cells and K562, but not fresh melanoma cells. In addition, allospecific E42 and C19 clones lysed neither K562 nor melanoma target cells. The finding of a clonal heterogeneity in the ability to lyse K562 or melanoma cells is in agreement with previous data on $CD3^+4^-8^-WT31^-$ unselected clones derived from peripheral blood (5). Although our present data do not provide information on the amplitude of the TCR-γ functional repertoire, this may be sufficiently wide to allow recognition of antigens present on allogeneic cells. In addition, it should be stressed that TCR-γ^+ alloreactive clones have been successfully derived from different individuals. Whether the recognized antigens are repre-

TABLE I

Cytolytic Activity of Allogeneic MLC-derived $CD3^+4^-8^-WT31^-$ Clones

Clone	^{51}Cr-labeled target cells				
	Autologous PHA blasts	Specific allogeneic PHA blasts	Nonspecific allogeneic PHA blasts	Fresh melanoma cells	K562
E 60	0*	22	0	11	10
E 90	0	44	8	36	100
E 42	0	20	0	2	4
E 93	0	34	1	59	100
C 11	0	16	ND	0	30
C 19	0	17	ND	0	0
M 14	0	19	ND	ND	ND

* Results are expressed as percent specific ^{51}Cr-release at a lymphocyte/target cell ratio of ~3:1.
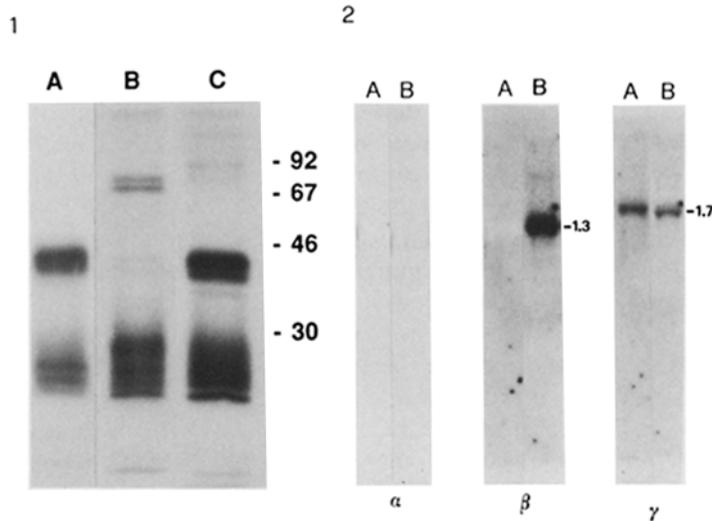
FIGURE 2.   (*1*) Immunoprecipitation and SDS-PAGE analysis of the CD3-associated molecules expressed in allogeneic MLC-stimulated CD3$^+$4$^-$8$^-$WT31$^-$ cells (*A* and *B*) and in the CD3$^+$4$^-$8$^-$WT31$^-$ clone 6.6.1. (*C*) Under nonreducing conditions (*B*) or under reducing conditions (*A* and *C*). (*2*) Northern blot analysis of TCR gene products in allogeneic MLC-stimulated CD3$^+$4$^-$8$^-$WT31$^-$ cells (*A*) and in the PEER cell line (*B*). The blots were hybridized to the TCR-α, -β, and -γ probes.

sented by MHC-encoded molecules remains to be determined. In a preliminary experiment with a TCR-γ$^+$ alloreactive clone, anti–class I but not anti–class II mAbs inhibited the specific lysis of allogeneic cells, when added to the cytolytic assay. Along this line, it has been reported that TCR-γ$^+$ cells isolated from alloimmunized BALB/c *nu/nu* mice may recognize class I MHC molecules (11).

Summary

These experiments were designed to define the ability of human TCR-γ$^+$ cells to recognize allogeneic cells. TCR-γ$^+$–enriched populations were obtained by treating peripheral blood E-rosetting cells with anti-CD4 and anti-CD8 mAbs. The resulting populations were CD2$^+$4$^-$8$^-$ expressed variable proportions of CD3$^+$ cells (40–90%), and did not react with the WT31 mAb, which is specific for a framework determinant of the α/β heterodimer that serves as receptor for antigen on most human T lymphocytes. After mixed lymphocyte culture with irradiated allogeneic cells for 7 d and 3 additional days in rIL-2 (100 U/ml), cells underwent proliferation in three of five individuals tested. In addition, MLC-derived cells lysed ^{51}Cr-labeled PHA-induced blasts derived from the allogeneic cells used as stimulator, but not allogeneic unrelated or autologous blast cells. No cytotoxicity against autologous or allogeneic target cells could be induced by culturing CD3$^+$4$^-$8$^-$WT31$^-$ lymphocytes in MLC with irradiated autologous cells. Surface iodination of allogeneic MLC-activated CD3$^+$4$^-$8$^-$WT31$^-$ cells followed by lysis in 1% digitonin and immunoprecipitation with anti-CD3 mAb indicated that the CD3-associated molecules consisted of a major 45-kD band and a minor band of 43 kD. Northern blot analysis

showed that mRNA for the γ chain was expressed at high levels, whereas mRNAs for α and β chains were missing. These data support the notion that TCR-γ rather than TCR-α/β is expressed in allospecific CD3$-4-8-$WT31$^-$ cell populations. Clones were further derived from MLC-stimulated CD3$^+$4$^-$8$^-$WT31$-$ populations. All the seven clones studied in detail maintained the surface phenotype as well as the cytolytic pattern of the original MLC populations, thus only specific allogeneic PHA-induced blasts were lysed. NK-sensitive as well as NK-resistant tumor targets were variably susceptible to lysis; therefore, specific cytolytic activity against allogeneic cells was not necessarily linked to the expression of MHC-nonrestricted cytotoxicity against tumor cells.

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