Brief Definitive Report

HUMAN AUTOLOGOUS MIXED LYMPHOCYTE REACTIVITY IS PRIMARILY SPECIFIC FOR XENOPROTEIN DETERMINANTS ADSORBED TO ANTIGEN-PRESENTING CELLS DURING ROSETTE FORMATION WITH SHEEP ERYTHROCYTES*

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Until recently, it was believed that only cells modified in their surface structure would stimulate autologous cells. Recent findings that human autoreactive T cells with exquisite specificity for unmodified non-T cells (NT cells) are readily demonstrable after changing the relative proportions between T and NT cells (1, 2) have therefore attracted much attention. These authors have shown that T cells positively enriched by their capacity to form rosettes with sheep erythrocytes (SRBC) will be induced to proliferate when cultivated with autologous NT cells. Subsequent studies (3–6) revealed specificity for self, the build-up of an immunological memory, and the generation of suppressor and killer cells in these autologous mixed lymphocyte reactions (AMLR). These observations led to the view that autoreactive T cells proliferating in AMLR represent members of a self-regulatory immune control system. Our search to define AMLR-inducing autoantigens led to quite contrary results: we present evidence that the majority of T cells proliferating in AMLR are not specific for autoantigens but are specific for xenoantigens that are derived from SRBC during the rosetting procedure.

Materials and Methods

Cell Separation Techniques. Peripheral blood mononuclear cells were isolated from heparinized blood of healthy volunteers by centrifugation on Ficoll-Isopaque (Lymphoprep, Nyegaard, Oslo, Norway) (4). T cells separated by rosette formation with SRBC (T_S cells) and NT cells separated by rosette formation with SRBC (NT_S Cells) were isolated by a second Ficoll-Isopaque gradient after rosette formation with neuraminidase-treated SRBC (4, 8). Alternatively, T and NT cells were enriched by discontinuous density gradient centrifugation on Percoll (Pharmacia, Uppsala, Sweden) using the methods described by Ulmer and Flad (7). 1×10^8 mononuclear cells were suspended in 11.25 ml Percoll solution with a density of 1.080 g/ml. The sample was subsequently overlayered with 7.5 ml of the following Percoll suspensions, exhibiting densities of 1.068, 1.066, 1.064, and 1.062 g/ml, respectively. Cells were

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brought to equilibrium by centrifugation at 390 g_{av} for 30 min at 18°C. T cells separated by centrifugation on Percoll gradients (T_P) representing 38 ± 3% of the total cell input were harvested from the interface between the 1.080 and 1.068 layers. NT cells separated by centrifugation on Percoll gradients (NT_P) comprising 37 ± 2% of the total input were harvested from the interface between the 1.066 and 1.064 layers. NT_P cells were separated into glass-adherent and glass-nonadherent cells, as previously described (8). Exposure to xenoproteins was performed by adding the same numbers of SRBC or the same concentration of fetal calf serum (FCS) that had been used above to separate NT cells from NT_S cells (4, 8). Incubation was performed at 37°C for 15 min. SRBC were removed from NT_P cells by centrifugation on Ficoll-Isopaque and FCS by three washes. The composition of the cell suspensions described above was as follows: T_S cells, 91 ± 4% T cells, <2% NAS-esterase$^+$ cells, and 3 ± 2% HLA-DR$^+$ cells; NT_S cells contained 11 ± 5% T, 55 ± 7% NAS$^+$, and 53 ± 12% HLA-DR$^+$ cells; NT_P cells contained 29 ± 9% T, 52 ± 11% NAS$^+$, and 46 ± 18% HLA-DR$^+$ cells; glass-adherent NT cells contained 4 ± 2% T cells, 83 ± 7% NAS$^+$, and 83 ± 3% HLA-DR$^+$ cells.

Autologous Mixed Lymphocyte Cultures. 1° AMLR cultures were established as previously described (8). 5×10^4 responder cells were incubated for 6 d with 1×10^5 mitomycin C-treated stimulator cells in a total volume of 150 µl of complete medium in round-bottomed microtiter plates (Titertek; Linbro Scientific Inc., Hamden, CT). Cells for 2° AMLR were cultivated under identical conditions. 50 µl of culture medium was renewed at day 7. Cells from the wells were pooled at day 10. 5×10^4 of these sensitized cells were restimulated, and proliferation was assessed at the peak of secondary reactivity from 36 to 48 h of culture.

Cell Marker Analyses. To identify monocytes, staining of cytocentrifuge smears for unspecific esterases was performed (8). T cells and HLA-DR$^+$ cells were identified using the pan-T-specific murine monoclonal antibody T 101 and a monoclonal murine anti-HLA-DR antibody NEI-011 (New England Nuclear, Boston, MA) in indirect immunofluorescence.

Results

Stimulation of T Cells by NT Cells Is Not Dependent upon the Relative Composition of the Stimulator Cells but upon Their Exposure to Xenogeneic Determinants. In a previous publication, we demonstrated that the presence of HLA-DR$^+$-adherent NT cells among autologous stimulators is essential to induce proliferation in AMLR (8). If, however, different preparations of NT cells containing identical numbers of HLA-DR$^+$ and NAS$^+$ cells were compared for their stimulatory capacity in AMLR, striking differences were observed. As shown in Fig. 1, only minimal T cell proliferation was induced by NT_P cells or almost pure preparations of monocytes. NT_P cells could, however, be readily converted into strong stimulators when briefly exposed to SRBC or FCS (Fig. 1).

T Memory Cells Generated in 1° AMLR Lack Specificity for Autologous Antigens but Exhibit Specificity for Xenogeneic Antigeneic Determinants. The immunological specificity generated in 1° AMLR has been analyzed using crosswise NT cells exposed to xenogeneic determinants as stimulators in 1° and 2° AMLR. Results are shown in Fig. 2. Significant secondary AMLR responses were exclusively observed when the stimulatory NT cells for both primary and secondary AMLR reactions had been exposed to xenogeneic sera or cells. This indicated that the immunological memory generated in these cultures is not directed against autoantigens but against xenogeneic determinants.

We also investigated the significance of the weak AMLR response seen in the absence of xenogeneic determinants. As shown in Fig. 2, the proliferative response against NT_P cells could not be further expanded by restimulation with NT_P cells. Moreover, peak proliferation in this combination was not seen after 2 d but rather after 5–6 d of 2° cultures (results not shown).

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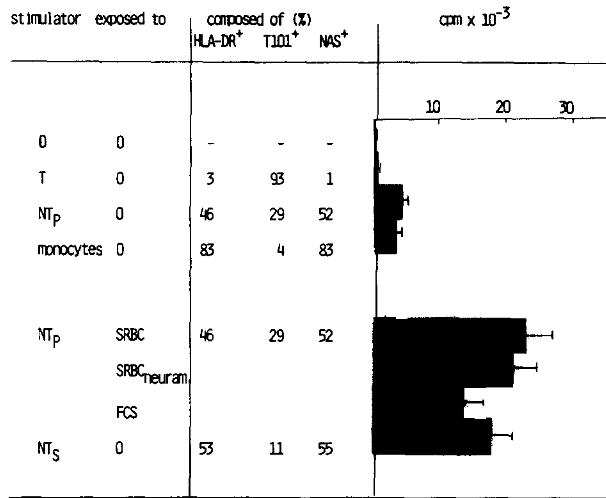

FIG. 1. Stimulation of T cells by NT cells is not dependent upon the relative composition of the stimulator cells but upon their exposure to the xenogeneic determinants. T cells (T_P cells in two experiments and T_S cells in two experiments) were stimulated with the various mitomycin C-treated autologous cell types indicated above. The numbers of HLA-DR^+ cells of T cells and of monocytes were assessed as described in Materials and Methods. NT_P cells were exposed to SRBC or FCS, as described in Materials and Methods. Proliferative activities on day 6 are given as mean ± SEM of all four experiments performed. No differences were observed between T_S and T_P cell response patterns.

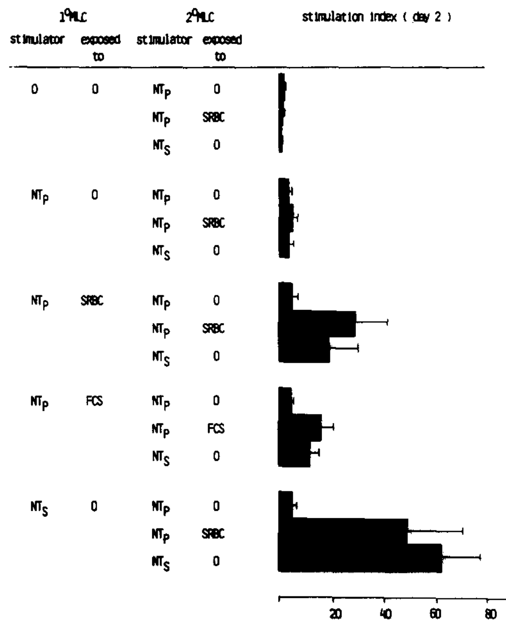

FIG. 2. T memory cells generated in 1° AMLR lack specificity for autologous determinants but exhibit specificity for xenogeneic determinants. T memory cells were stimulated in 1° AMLR with autologous NT cells that had or had not been exposed to xenogeneic determinants. Exposure to xenoproteins was performed as described in Materials and Methods. After 10 d of culture, each of the sensitized cell types was restimulated with xenoprotein-exposed or -unexposed NT cells. Proliferative capacities on day 2 are given as mean ± SEM of stimulation indices of all four experiments performed.

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TABLE I

T Memory Cells Generated in 1° AMLR Are Specifically Restimulated by NT_P Cells Pulsed with Xenogeneic Sera

1° AMLR responder:stimulator	2° AMLR stimulator exposed to*		Proliferation −cpm × 10^{-3}‡	
			Day 2	Day 4
^AT_P:^ANT_S§	^ANT_P	NSS 30 mg/ml	5.8	3.2
		15 μg/ml	0.8	−0.5
		NGPS 30 mg/ml	4.8	2.6
		15 μg/ml	0.7	−0.7
		NRS 30 mg/ml	3.6	2.1
		15 μg/ml	0.6	−0.2
		FCS 30 mg/ml	21.2	19.6
		15 μg/ml	0.4	−0.7
	^BNT_P§	NSS 30 mg/ml	2.1	0.9
		5 μg/ml	1.1	−0.2

* NT_P cells at a concentration of 1 × 10^6 cells/ml RPMI 1640 supplemented with Hepes L-glutamine and antibiotics were incubated for 30 min with heat-inactivated normal sheep serum (NSS), normal guinea pig serum (NGPS), normal rabbit serum (NRS), or FCS. Subsequently, xenoserum-pulsed cells were washed three times in the same medium and were added to T memory cells, as described in Materials and Methods.

‡ Differences between T memory cell proliferation induced by xenoserum-pulsed NT_P and human pool serum-pulsed NT_P cells are given. Values for ^AT_P:^ANT_S on day 2 were 1.0 and on day 4, 0.8; values for the same cells restimulated with human serum-pulsed autologous NT_P cells (A_{NT}) were 1.5 on day 2 and 3.7 on day 4; values for the same cells restimulated with allogeneic NT_P cells restimulated with allogeneic NT_P (B_{NT}) were 3.7 on day 2 and 15.6 on day 4.

§ Individuals A and B represent unrelated individuals.

T Memory Cells Generated in 1° AMLR Can Be Restimulated by Autologous NT Cells That Have Been Pulsed with Xenogeneic Serum Proteins. The finding of extensive cross-reactions in restimulation experiments of T cells sensitized to different xenoantigens, such as FCS and SRBC, suggested that SRBC-associated serum proteins exhibiting cross-antigenicity among the various mammalian species represent the main antigenic determinants involved. To test for this assumption, T cells were sensitized in 1° AMLR to NT_S cells and were subsequently restimulated with NT_p cells that had or had not been pulsed with human or xenogeneic mammalian sera. Results of one typical experiment are shown in Table I. Whereas restimulation of T memory cells sensitized in AMLR was never achieved by NT_p cells pulsed to human sera, significant restimulation was readily demonstrated with NT_p cells pulsed with any of the xenogeneic sera tested. Moreover, serum-derived xenoantigens were more efficiently presented to T memory cells when associated with autologous NT cells than with allogeneic NT cells.

Discussion

The most crucial question on the biological role of AMLR concerns the nature of the target antigen(s) involved in the induction of this reactivity. One clue to their further definition was obtained in experiments indicating that the recognition of self in AMLR requires identical genetical prerequisites as the recognition of foreign antigens in association with self (8, 10–12). We have therefore searched for the possibility that foreign determinants recognized in association with self-Ia are the target antigens in AMLR. Our findings strongly support the view that the vast majority of T cells proliferating in human AMLR exhibit specificity for xenoproteins and that this reactivity is restricted for self by the HLA-DR type of antigen-presenting cell.

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The question of the effect of xenoprotein exposure on AMLR has already been addressed in previous studies. In mice, strong proliferative responses were exclusively observed in the presence of FCS or polyethylene glycol (10, 11). Strong AMLR in the absence of xenogeneic determinants was demonstrated in guinea pigs using peritoneal exudate macrophages as stimulators (12). Because exudate cells were by far the best stimulators, it is difficult to decide whether stimulation represents an intrinsic capacity of macrophages or a consequence of modification by inflammatory processes (12). In man, weak AMLR reactivity of T cells was induced by unseparated peripheral blood mononuclears (1, 2). Because FCS was used in washing buffers (2) and because T cells still contaminated with substantial numbers of Ig^+ mononuclears were separated by exposure to xenogeneic determinants (1, 2), it is not possible to exclude the participation of foreign determinants in these experiments.

Summary

We present evidence that most T cells proliferating in response to autologous sheep erythrocyte (SRBC)-separated non-T cells (NT) cells are not specific for autoantigens but for antigens derived from xenogeneic sources. The conclusion was based on the following three observations. First, we found that NT cells isolated in the absence of xenoproteins by means of density gradient centrifugation on Percoll only weakly stimulated autologous T cells. Because this weak proliferation could not be expanded in restimulation experiments, its significance as an immune recognitive event remains questionable. NT cells isolated by the above method in the absence of xenogeneic determinants readily acquired stimulatory capacity after brief exposure to either SRBC or fetal calf serum. Second, restimulation of T memory cells generated in 1° autologous mixed lymphocyte reaction (AMLR) against SRBC-separated autologous NT cells was exclusively seen when NT cells exposed to or separated with xenoproteins were used for restimulation. Third, T memory cells generated against SRBC-separated autologous NT cells were specifically restimulated by autologous Percoll-separated NT cells that had been pulsed with a variety of xenogeneic mammalian sera. These xenogeneic determinants were preferentially recognized in context with autologous $HLA\text{-}DR^+$ cells. From these findings and from our previous results that indicated an absolute requirement of $HLA\text{-}DR^+$-adherent NT cells (8), we conclude that human AMLR primarily does not represent an autoantigen but a xenoantigen response that is genetically restricted by the HLA-DR type of the antigen-presenting cell.

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