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IgD-RECEPTOR-POSITIVE HUMAN T LYMPHOCYTES

I. Modulation of Receptor Expression by Oligomeric IgD and Lymphokines

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Studies with human myeloma-derived IgD have demonstrated the existence of IgD-R on peripheral blood T cells. These receptors, which are detected by rosetting with IgD-coated ox E (IgD-rosette-forming cells), are competitively inhibited by IgD, but not by IgM or IgG. Similar results were obtained with human T cell clones and T hybridomas derived from such clones either by rosetting assays or by staining with biotinylated-IgD. In agreement with studies of murine IgD-R cells, human IgD-R can be up-regulated by exposure of peripheral blood T cells, T cell clones, and hybridomas derived from such clones, to oligomeric IgD, but not monomeric IgD. Human IgD-R can also be induced by IL-2, IL-4, and IFN-γ. In contrast with studies of murine IgD-R, which are expressed primarily by CD4+ cells, phenotyping studies show that both the CD4+ and CD8+ human T cell subsets are capable of expressing IgD-R.

IgD is present on the surface of the majority of B lymphocytes, whereas, serum IgD levels are low. It has been suggested by others that the major function of IgD is to serve as a surface receptor [1]. It is now clear that IgM and IgD differ with respect to their relative ability to transmit inhibitory signals to the B cell [2] and to some extent also with regard to the molecules to which they are linked in the cell membrane [3]. However, no definitive role different from B cell membrane IgM has been found for IgD. Evidence for an immunoregulatory role of IgD was obtained from our studies in mice that demonstrated that injection of myeloma-derived IgD into eu- and R expression resulting from exposure of T cells to mye- thymic, but not athymic mice, caused significant en- enhanced humoral immune responses to various Ag [4]. Studies of the cellular basis for this effect have led to the identification of T8+ cells in mice [5, 6]. Increased IgD-R expression resulting from exposure of T cells to mye- loma IgD, either in vivo or in vitro, correlated with in- creased Th cell activity [5]. Up-regulation of these recep- tors was also observed after in vivo or in vitro exposure of T cells to lymphokines including IL-2, IL-4, and IFN-γ, or to cross-linked B cell membrane IgD [7-9].

Studies with other Ig-R, including those specific for IgG, IgA, IgE, and IgM, have shown these receptors to be specific for the Fc region of the Ig molecule. Investigation of the fine specificity of murine IgD-R were conducted using mutant IgD molecules lacking specific C3 domains. These studies revealed that each of the murine 6 H chain domains can bind independently to IgD-R [10]. Enzymatic deglycosylation of N-linked IgD glycans, followed by isolation of the IgD carbohydrate fraction, allowed us to directly examine the contribution of these sugars to the binding of IgD to IgD-R. Competitive inhibition experiments, using these IgD-associated carbohydrates as well as specific sugars, in particular N-acetylgalactosamine, have provided strong evidence that murine IgD-R are lectins which bind to N-linked, IgD-associated carbohydrates [4].

In the present study we investigated the possibility that IgD-R+ T lymphocytes also exist in man. Inasmuch as we were able to demonstrate murine IgD-R using a rosetting technique, we used a similar approach in our search for human IgD-R. Our results show that, in agreement with studies with murine IgD-R, human T lymphocytes can be induced to express IgD-R in response to various stimuli, including oligomeric IgD and certain lymphokines. However, in contrast to murine IgD-R that appear to be expressed only on CD4+ cells, both CD4+ and CD8+ human T cell subsets were found to express these receptors.

MATERIALS AND METHODS

Purification of IgD. Human IgD myeloma sera were generously provided by Dr. B. Pernis (Columbia University College of Physicians and Surgeons, New York, NY), Dr. F. D. Finkelman (Uniformed Services University of Health Science, Bethesda, MD) and Dr. I. Mac Leman (Department Immunology, University of Birmingham Medical School, Birmingham, UK). Purification of murine IgD was achieved by affinity-purification with the use of goat-anti-human IgD (Tago, Inc., Burlingame, CA) coupled Sepharose 4B. In some experiments, isolated IgD was chemically aggregated by treatment with glutaraldehyde (Sigma Chemical Co., St. Louis, MO) as described [10]. mAb and polyclonal antibodies. B cell hybridomas producing mAb OKT4 (anti-CD4) and OKT8 (anti-CD8) were obtained from the American Type Culture Collection (Rockville, MD). Hybridomas were maintained in pristane-primed BALB/c mice (Charles River Labora- tory, Charles River, NY). FITC- and phycoerythrin-conjugated mAb specific for CD3, CD4, CD8, and CD20 were obtained from Coulter Immunology, Inc. (Hialeah, FL). Affinity-purified goat anti-mouse Ig

was purchased from Boehringer-Mannheim (Indianapolis, IN). Cytotoxic antibody cocktails (Lymphokwick) designed to isolate either T lymphocyte populations (CD4+ or CD8+) or B lymphocytes were obtained from One Lambda, Inc. (Los Angeles, CA).

Isolation of PBL subsets. Samples of heparinized human blood from normal donors were obtained either from the Cellular Components Division of New York Blood Center (New York, NY) or from healthy volunteers at NYU Medical Center (New York, NY). Mononuclear cells were isolated by Ficol-Hypaque density gradient centrifugation as described (11). Lymphocyte T cell subpopulations were fractionated either by direct panning of PBL on petri dishes coated with mouse mAb specific for CD4 or CD8, or by indirect panning of mononuclear cell subpopulations. CD4+-treated PBL on goat anti-mouse IgG-coated dishes. Alternatively, PBL were treated with specific Lymphokwick cytotoxic cocktails (One Lambda, Inc., Los Angeles, CA) to isolate B cells, CD4+, or CD8+ T cells. The purity of isolated lymphocyte populations was confirmed by cytofluorographic analysis with an Elite Flow Cytometer ( Coulter, Hialeah, FL) using FITC-labeled, CD-specific mAb.

Human T cell lines. The tetanus toxoid-specific humaa CD4+ T cell clone, H8, and a T cell hybridoma derived from this clone (H9-CEM1) were the generous gift of Dr. D. Umetsu (Stanford University, School of Medicine, Stanford, CA). Several H9-CEM1 clones (i.e., H9-CEM1.5 and H9-CEM1.8) were subsequently established in our laboratory by limiting dilution of the H9-CEM1 line.

Lymphokines. Human rIL-2, IL-4, and IFN-γ were obtained from Genzyme Inc. (Boston, MA), and Genentech, Inc. (South San Francisco, CA), respectively. IgD-RFC assay. Ox RBC (Colorado Serum Co., Denver, CO) were coated with purified human IgD-myeloma protein or BSA using the CrC3J coupling method of Pastore (13). Such IgD- or BSA-coated indicator cells were then used to enumerate lymphocytes forming IgD- or BSA-RFC, respectively, using a modified rosetting assay previously described for murine T3 cells (5). Briefly, 0.2 ml of 1% indicator cells (in RPMI 1640 containing 2% FCS) was mixed with 0.1 ml of assay cells (at 2.5 × 106/ml) for 30 min at 37°C. The cell mixtures were then centrifuged at 500 rpm (200 × g) for 3 min and stored in pellets at 4°C for periods ranging from 1 h to overnight. Immediately before scoring rosettes, the lymphocytes were stained with addition of 0.1% toluidine blue. Although murine- or daisy-type rosettes were generally observed, lymphocytes surrounded by more than three indicator cells were also scored as rosettes. In some experiments, the ability of human IgG, IgM (Boehringer-Mannheim) or IgD myeloma protein to competitively inhibit IgD-RFC was investigated. This was achieved by supplementing the 0.3 ml rosetting mixture with 0.05 ml of PBS containing a range of concentrations of the inhibitors and incubating on ice for 15 min. prior to the 30-min incubation at 37°C.

Biostaining of human IgD. Human IgD was biotinylated using a modification of a previously described method (14). Briefly, 1 mg of aggregated IgD in 0.1 M HEPES, pH 8.0, was incubated at 4°C for 4 h with 1 mg of biotin-N-hydroxysuccinimide ester (Calbiochem-Behring, La Jolla, CA). After biotinylation, free N-hydroxy-succinimide modification of a previously described method (14). Briefly, 1 mg of aggregated IgD in 0.1 M HEPES, pH 8.0, was incubated at 4°C for 4 h with 1 mg of biotin-N-hydroxysuccinimide ester (Calbiochem-Behring, La Jolla, CA). After biotinylation, free N-hydroxy-succinimide and uncoupled biotin was removed by extensive dialysis of the biotylated-IgD in PBS containing 100 mM HEPES and 0.056% NaN3, pH 8.0.

RESULTS

IgD-mediated Up-regulation of IgD-R. We have previously shown that murine IgD-R can be up-regulated by polymerized or cross-linked IgD, but not by monomeric IgD (8). Reports concerning FcR for IgA and IgE isotypes have also shown the need for oligomeric forms of these Ig isotypes in the up-regulation of FcαR and FcεR, respectively (15, 16). In our initial attempts to identify IgD-R on human PBL, we therefore compared the ability of non-aggregated monomeric IgD with that of glutaraldehyde-aggregated IgD to induce these receptors. Examination of untreated PBL from normal human individuals revealed a very low frequency of cells capable of forming rosettes with IgD-coated ox RBC (Table I). However, oligomeric human IgD myeloma protein effectively up-regulated IgD-R expression by PBL after an 18-h incubation period. The monomeric form of this IgD myeloma protein only induced a small increase in IgD-R expression by PBL, with frequencies of IgD-RFC less than half that observed for cells exposed to the aggregated form. Inasmuch as murine IgD-R have been shown to be optimally up-regulated on splenic T cells after a 1-h incubation period with oligomeric IgD (5, 6), shorter PBL incubation periods were also tested. Exposure of human PBL for 1 h to aggregated, but not monomeric IgD, resulted in suboptimal induction of IgD-R as compared with an 18-h incubation period (Table I). PBL cultured in the presence of glutaraldehyde-aggregated human IgG failed to show a significant increase in the expression of IgD-R. Control RFC assays with BSA-coated ox RBC were performed in all experiments. Essentially the same low BSA-RFC values (1 to 2%) were observed under all experimental conditions tested.

Optimal induction of IgD-R expression by human PBL was observed after an 18-h incubation period with aggregated IgD concentrations >25 μg/ml (Fig. 1). Similar dose response curves were obtained using either soluble or insolubilized forms (i.e., coated dishes) of IgD.

Induction of human IgD-R by lymphokines. Inasmuch
as studies in mice have shown that IgD-R can be up-regulated by in vivo or in vitro exposure of T cells to lymphokines (7, 17), we performed similar in vitro experiments with human PBL. As shown in Figure 2, a significant increase in the frequency of IgD-RFC was observed after overnight incubation of either whole PBL, or CD3+ peripheral blood T cells with IL-2 or IL-4. In agreement with results concerning the regulation of murine IgD-R by these lymphokines, synergy between IL-2 and IL-4 was not observed. IFN-γ was also found to induce human IgD-R (Fig. 2).

Numerous studies have made it clear that triggering of the CD3/T cell Ag receptor complex results in a series of metabolic events that lead to T cell activation (18). It was therefore of interest to determine whether anti-CD3 activation of T cells would also up-regulate the expression of IgD-R. As shown in Figure 2, overnight stimulation of PBL with insolubilized mAb (anti-CD3), caused a significant increase in the frequency of IgD-RFC. Bioassays for IL-2 were performed using supernatants derived from these overnight anti-CD3-stimulated PBL cultures. No detectable IL-2 activity was observed. Recent experiments with anti-CD3-stimulated purified T cells have yielded similar results (data not shown) suggesting that costimulation (e.g., by APC) is not required for up-regulation of IgD-R expression.

Specificity of IgD-RFC. Competitive inhibition experiments were performed to investigate the specificity of IgD-RFC. As shown in Figure 3, when PBL were induced to express IgD-R after an 18-h incubation period in medium supplemented with 50 μg/ml aggregated IgD, 1000 U/ml IL-2, or 100 U/ml IL-4, the frequency of IgD-RFC increased from background values of 1 to 2% to 24 to 26%. A significant, dose-dependent competitive inhibition of IgD-RFC was observed when unaggregated isolated IgD was added to the rosetting mixture at the initiation of the rosetting assay. Complete inhibition of IgD-RFC (to background levels) was found using IgD concentrations ≥50 μg/RFC mixture.

Similar competitive inhibition experiments performed with equivalent concentrations of human IgG or IgM revealed no inhibition of IgD-RFC (IgD-RFC values of 24 to 25%). This was true regardless of the stimulus used to induce IgD-R. Thus, the human and murine IgD experimental systems are again similar, because IgD-R on murine T cells are also effectively blocked by monomeric IgD, and not by other Ig isotypes (5).

Phenotype of human T6 PBL. As mentioned earlier, in agreement with results obtained in studies with mice, human IgD-R+ PBL that respond with increased IgD-R expression are also CD3+ T cells (Fig. 2). Moreover, we have previously shown that murine T cells belonging to the CD4 Th cell subset (L3T4+), but not CD8+ cytotoxic/suppressor T cells, are capable of expressing these receptors (5). When isolated CD4+ and CD8+ human T cell subpopulations were examined for their ability to display increased IgD-R expression after stimulation with 50 μg/ml IgD or 1000 U/ml IL-2, both populations responded with increased IgD-RFC frequencies (Table I). Thus, in contrast with the murine IgD experimental system, human IgD-R+ T cells are phenotypically heterogeneous.

Expression of IgD-R by human T cell lines. Resting
murine T cell clones were previously shown to express low levels of IgD-R. When stimulated with Ag in the absence of IgD, IgD-R expression increased significantly (7). Indeed, this latter effect provided the first suggestion that lymphokines could regulate the expression of these receptors. Certain CD4+ T cell hybridomas, including the pigeon cytochrome C-specific line, 2H10, were found to express IgD-R constitutively (17). Such cells proved invaluable in studies of soluble IgD-binding factor, released by such cells after their stimulation with IL-2 (19).

We investigated the expression of IgD-R on the tetanus toxoid-specific CD4+ human T cell line, H9 (12). In the present study, only 1 to 2 wk rested H9 cells were used.

As shown in Table III, mean background IgD-RFC values for unstimulated H9 cells were 11% ± 3.1. When cultured for 18 h in the presence of 50 μg/ml aggregated IgD, or 1000 U/ml IL-2, IgD-RFC values increased to 26% ± 1.7 and 20% ± 1.7, respectively. A T cell hybridoma (H9-CEM1), derived from the H9 clone, was also tested for IgD-R expression. The CEM1 T lymphoma fusion partner used to prepare these hybridomas was found to express low background levels of IgD-R (IgD-RFC values ≤2%). No change in the frequency of IgD-RFC was observed after stimulation of CEM1 cells with IgD or IL-2 (data not shown). In contrast, the H9-CEM1 hybridoma line responded to aggregated IgD (higher IgD-RFC), although the response to IL-2 was low. Selected clones derived from this hybridoma gave higher responses (Table III: H9-CEM1.5 and H9-CEM1.8). In agreement with studies of murine T hybridoma cells, we found that the characteristic IgD-R expression patterns of T cell hybridomas are lost with continuous culture periods >1 mo. This instability is overcome, however, by routine cryopreservation of established clones showing particular IgD-R expression patterns.

| Inducing Agent | Responding Cells | Percent IgD-RFC (Mean ± SD)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>CD3+</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>None</td>
<td>CD4+</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>None</td>
<td>CD8+</td>
<td>ND</td>
</tr>
<tr>
<td>Aggregated IgD</td>
<td>CD3+</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Aggregated IgD</td>
<td>CD4+</td>
<td>18 ± 0.5</td>
</tr>
<tr>
<td>Aggregated IgD</td>
<td>CD8+</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>IL-2</td>
<td>CD3+</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>IL-2</td>
<td>CD4+</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>IL-2</td>
<td>CD8+</td>
<td>ND</td>
</tr>
</tbody>
</table>

* A total of 50 μg of aggregated IgD was allowed to adhere to a 60-mm petri dish and 107 cells were incubated in this dish for 18 h at 37°C.

* Negatively selected by complement-mediated cytolysis (experiments 1 and 2) or positively selected by indirect panning (experiment 2). The purity of each population was assessed by flow microfluorimetry analysis and ranged from 96 to 99%.

* RFC assays performed in triplicate.

As shown in Table III, mean background IgD-RFC values for unstimulated H9 cells were 11% ± 3.1. When cultured for 18 h in the presence of 50 μg/ml aggregated IgD, or 1000 U/ml IL-2, IgD-RFC values increased to 26% ± 1.7 and 20% ± 1.7, respectively. A T cell hybridoma (H9-CEM1), derived from the H9 clone, was also tested for IgD-R expression. The CEM1 T lymphoma fusion partner used to prepare these hybridomas was found to express low background levels of IgD-R (IgD-RFC values ≤2%). No change in the frequency of IgD-RFC was observed after stimulation of CEM1 cells with IgD or IL-2 (data not shown). In contrast, the H9-CEM1 hybridoma line responded to aggregated IgD (higher IgD-RFC), although the response to IL-2 was low. Selected clones derived from this hybridoma gave higher responses (Table III: H9-CEM1.5 and H9-CEM1.8). In agreement with studies of murine T hybridoma cells, we found that the characteristic IgD-R expression patterns of T cell hybridomas are lost with continuous culture periods >1 mo. This instability is overcome, however, by routine cryopreservation of established clones showing particular IgD-R expression patterns.

<table>
<thead>
<tr>
<th>Inducing Agent</th>
<th>H9 clone</th>
<th>H9-CEM1</th>
<th>H9-CEM1.5</th>
<th>H9-CEM1.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6 ± 1</td>
<td>13 ± 2</td>
<td>10 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Aggregated IgD</td>
<td>22 ± 1</td>
<td>20 ± 3</td>
<td>36 ± 0.2</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>16 ± 0.2</td>
<td>31 ± 0.1</td>
<td>19 ± 7</td>
</tr>
</tbody>
</table>

* Cells (2.5 × 106/ml) were incubated for 18 h in medium ± glutaraldehyde-aggregated IgD (50 μg/ml) or IL-2 (1000 U/ml). RFC assays performed in triplicate. BSA-RFC percentages were <1 (n = 4) for H9 clone, 4.3 ± 3 (n = 6) for CEM 1.11.9 ± 3.5 (n = 6) for CEM1.5, and 6.8 ± 4.7 (n = 5) for CEM1.8.
their interaction with specific carbohydrate moieties as
T6 cells and increased ability of such cells to transfer

Figure 4. Staining of IgD-induced H9-CEM1 cells with biotinylated-
IgD. H9-CEM1 cells [5 x 10^6] were incubated for 18 h in 2 ml of complete
RPMI 1640 medium ± 50 μg/ml aggregated IgD. The cells were washed
twice in PBS containing 1% BSA and 0.05% NaN3. The 10^6 cells in 50 μl
were stained for 30 min at room temperature with 50 μg of biotinylated-
IgD, washed twice, and then incubated on ice for 30 min with avidin-
FITC. Control cells represent IgD-induced cells stained with avidin-FITC
only. Cells were analyzed cytofluorimetrically using a Coulter Elite flow
cytometer (Coulter Electronics, Hialeah, FL).

DISCUSSION

Our studies with murine IgD have strongly suggested
that, in addition to its role as a B cell membrane receptor
for Ag, IgD may also play a role in the regulation of
immune responses via its recognition of IgD-R' immu-
naugmenting Th cells [9]. These studies have shown a
correlation between up-regulation of IgD-R expression
by T6 cells and increased ability of such cells to transfer
helper activity for antibody responses [6]. More recently,
investigation of the fine specificity of murine IgD-R has
indicated that these IgD-specific receptors are lectins that
bind to N-linked IgD-associated glycan molecules. This
observation, together with our previously reported func-
tional data for murine T6 cells, has led us to speculate
that up-regulation of IgD-R on Th cells may facilitate
their interaction with specific carbohydrate moieties as-
associated with B cell membrane IgD. In the present study,
we investigated the possibility that human IgD-R' T
lymphocytes also exist in man. Our results indicate that
these receptors can be detected by a modified rosetting
technique previously described for murine IgD-RFC or by
staining using biotinylated aggregated IgD. Moreover, hu-
man IgD-R expression is up-regulated by stimuli known
to induce IgD-R on murine T cells. The similarity extends
everywhere itself to the requirement that IgD needs to be cross-linked
for optimal stimulation of increased IgD-R expression on
human CD3^+ T lymphocytes.

The degree of aggregation required was not addressed
in the present studies. However, we have noted that an
occasional human myeloma IgD preparation causes up-
regulation of IgD-R without prior aggregation with gluta-
raldehyde. This suggests that oligomeric molecules suf-
face, as is the case for murine IgD, where dimeric IgD

myeloma protein (TEPC-1017) causes up-regulation with-
out the need for any further aggregation [8]. Moreover, in
more recent studies it was found that the soluble form of a
chimeric mouse-human IgD with anti-dansyl activity
(kindly provided by Dr. Sherri Morrison, UCLA, Los An-
egles, CA) causes up-regulation of IgD-R on human T
cells only in the presence of dansyl-protein conjugates,
i.e., as an immune complex. Thus, the biologic signifi-
cance of the need for cross-linking of IgD in terms of IgD-
R up-regulation may be that surface IgD on B cells needs
to be cross-linked for this induction to occur in vitro and
in vivo, as was previously shown in the murine system
[8, 9].

Examination of the kinetics of induction of human IgD-
R' lymphocytes revealed that unlike murine T6 cells,
which exhibit maximal expression of IgD-R after 90 min
of exposure to oligomeric IgD [6], up-regulation of IgD-R
expression by human T cells requires longer incubation
periods (18 h). Inasmuch as studies in the mouse have
focused on splenic and lymph node T cell populations,
rather than PBL, the need for extended incubation
periods to up-regulate IgD-R on human T cells may reflect
inherent differences in the T cell compartments studied.
The kinetics of induction of IgD-R expression by murine
peripheral blood T cells has not yet been investigated.

Although most of the current data concerning human
IgD-R generally parallels previously reported findings
with murine IgD-R' T lymphocytes, clear differences ex-
ist with respect to the phenotype of human T6 cells. Both
CD4^+ and CD8^+ human T cell subsets express IgD-R,
whereas, in the mouse only the CD4^+ T cell subset ex-
press IgD-R. The augmented helper cell activity of murine
T6 cells [5] is consistent with their CD4 phenotype. The
functional properties of the heterogeneous human IgD-
R' T cell populations identified in this study remain to
be investigated. It will be of interest to determine whether
isolated CD4^+, IgD-R' human T cells potentiate in vitro
antibody responses more effectively after IgD-mediated
up-regulation of IgD-R expression as compared with con-

control CD4^+ T cell clones. Similarly, the immunoregulatory
properties of IgD-R' CD8^+ T cells need to be examined.

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