Intrathymic clonal deletion is thought to be the major mechanism responsible for tolerance to nonsequestered antigens such as the ones expressed by bone marrow-derived cells. In the case of sequestered antigens that potentially do not come in contact with T cells in the thymus, it is thought that autoreactive T cells are present in periphery but are tightly regulated to prevent autoimmune diseases. Indeed, autoreactive T cells to sequestered antigens can be isolated in healthy individuals. However, the presence of autoreactive T cells to nonsequestered circulating antigens had not been observed. In this report, we present evidence for the presence, in the periphery of all healthy individuals tested (n = 25), of autoreactive T cells to GpIIb-IIIa, a membrane antigen present on bone marrow-derived cells that is expressed on circulating platelets and on the cell surface of the epithelial cells of the thymic stroma early in intrauterine life. Using an in vitro T-cell proliferation assay, we have demonstrated that activation of these specific GpIIb-IIIa autoreactive αβTCR+ CD4+CD8– T cells requires internalization and processing of the GpIIb-IIIa by antigen-presenting cells and its presentation by HLA-DR class II molecules in the presence of exogenous interleukin 2 (IL-2). This indicates that some autoreactive T cells directed against membrane antigens present on bone marrow-derived cells and also expressed in the thymus are not necessarily eliminated by intrathymic deletion.

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AUTOREACTIVE T CELLS to self antigens have been isolated not only from peripheral blood of individuals with autoimmune diseases but also from healthy individuals.1,2 For example, investigators have isolated autoreactive T cells to the myelin basic protein in individuals with or without multiple sclerosis.3,4 Other investigators have shown the presence of autoreactive T cells specific for type II collagen in patients with rheumatoid arthritis and in healthy individuals.5 These autoreactive T cells isolated from healthy individuals are directed against sequestered antigens that do not come in contact with T cells in the thymus. In the case of nonsequestered antigens, which can be present in the thymus during the process of thymic education, autoreactive T cells are hypothesized to be deleted to prevent autoimmune reactions.

GpIIb-IIIa, also designated αmβ3 integrin or CD41/CD61, is a heterodimeric surface membrane receptor for soluble adhesive proteins that is expressed abundantly as early as the 16th week of intrauterine life on megakaryocytes, platelets, and on epithelial cells of the thymic stroma.6 The GpIIb-IIIa complex is suspected to be the principal target in autoimmune thrombocytopenia (ATP).7,8 Recently, autoreactive T cells to GpIIb-IIIa have been isolated from individuals with ATP but not from healthy individuals as would be expected for a nonsequestered antigen.9,10

We have recently reported that in response to purified GpIIb-IIIa peripheral blood, T cells from healthy individuals express interleukin 2 receptor α (IL-2R-α) on their cell surface and secrete interferon (IFN)-γ, but they do not produce IL-2.11 Proliferation of these T cells occurs only in the presence of exogenous IL-2, IL-7, or IL-12.12,13 Thus, these T cells represent putative autoreactive T cells to a nonsequestered antigen. In the present study, we substantiate this possibility by demonstrating that response to GpIIb-IIIa results from its presentation to αβTCR+ CD4+CD8– by antigen presenting cells (APC) via the HLA class II pathway.

To do so, we have set up an in vitro assay to dissect the immune response to dioleoylphosphatidyl choline/dioleoylphosphatidyl serine (DOPC/DOPS) liposome-encapsulated GpIIb-IIIa using peripheral blood mononuclear cells (PBMC) from 25 different healthy individuals without detectable anti-GpIIb-IIIa antibodies. The results indicated that T cells originating from all healthy individuals tested proliferated in the presence of GpIIb-IIIa and exogenous IL-2, but the stimulation index was heterogeneous between healthy individuals. Proliferation was specific to natural GpIIb-IIIa and could not be attributed to fibrinogen, which is present in our GpIIb-IIIa preparations, to DOPC/DOPS liposomes, to alloantigens present on GpIIb-IIIa, or to neoantigens obtained during the process of GpIIb-IIIa purification. Furthermore, proliferation was found to be dependent on the GpIIb-IIIa antigen being internalized and processed by APC, presented by HLA-DR class II molecules, and recognized by αβTCR+ CD4+CD8– T cells. These results strongly suggest that GpIIb-IIIa autoreactive αβTCR+ CD4+CD8– T cells are present in the periphery of most, if not all, individuals and could be downregulated in individuals that have not developed anti-GpIIb-IIIa autoantibodies. This is the first evidence that autoreactive T cells to a human nonsequestered antigen expressed on bone marrow-derived cells and present during the process of thymic education can be found in the peripheral blood of healthy individuals.

MATERIALS AND METHODS

GpIIb-IIIa antigen. GpIIb-IIIa was purified from outdated platelet concentrates as previously described.15 GpIIb-IIIa was used in
0.02% Triton X-100 solubilized form or encapsulated in DOPC/DOPS liposomes (Avanti-Polar lipids, Alabaster, AL) at a molar ratio of 4:1. Liposomes were prepared as previously described.

Control DOPC/DOPS liposomes containing no GpIIb-IIIa were prepared by extension of multimembrane vesicles through 100 nm pore size polycarbonate filters (Nuclepore, Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Vancouver, Canada).

Cells. Human PBMC from healthy individuals without detectable GpIIb-IIIa antibodies were isolated by Ficoll-Hypaque density-gradient centrifugation of whole blood. Presence of anti-GpIIb-IIIa antibodies in the sera was assessed by two techniques: immunofluorescence on platelets pretreated with chloroquine and by monoclonal antibody-specific immobilization of platelet antigens. In some experiments, purified T cells were used. These cells were recovered by negative selection using the Immuan separation system (purity >95%; Biotecx, Houston, TX).

Proliferation assay. The proliferation assay was based on previously described techniques to clone T cells with minor modifications. PBMC were seeded at 1.5 x 10^6 cells/well in AIM-V serum-free lymphocyte medium (GIBCO-BRL, Gaithersburg, MD) with liposome-encapsulated GpIIb-IIIa in 96-well round microplates at 37°C, 5% CO₂. At day 7, IL-2 (50 U/ml) or liposomes were added and incubation continued for 3 more days, at which point cellular proliferation was measured by 3H-thymidine incorporation (1.0 μCi/mL; Amersham, Oakville, Ontario, Canada) during the last 16 to 18 hours. Cells exposed to 3H-thymidine were harvested on glass fiber filters and incorporation of the radioactive label was measured by liquid scintillation counting. Cells were harvested by trypsinization and the radioactivity was measured by liquid scintillation counting. PBMC of three healthy individuals without anti-GpIIb-IIIa antibodies were stimulated by DOPCDOPS liposomes and not by PBS. At higher concentrations, liposome-encapsulated GpIIb-IIIa gave a higher stimulation index than Triton X-100 solubilized GpIIb-IIIa, but at a lower concentration (1.25 to 2.5 μg/mL) both forms of GpIIb-IIIa gave the same stimulation index. This could be due to cytotoxicity of Triton X-100 at higher concentrations. Removal of Triton X-100 resulted in the formation of GpIIb-IIIa insoluble aggregates that did not induce proliferation.

Analysis on SDS-PAGE and by ELISA indicated that our GpIIb-IIIa preparations were contaminated with variable amounts of fibrinogen (1% to 50%) (data not shown). It has been shown that fibrinogen could have a mitogenic effect on early hematopoietic precursors, CD34-enriched cell populations and monocytes. We have tested the possibility that T cells were stimulated by DOPCDOPS liposomes and not by GpIIb-IIIa in our in vivo assay. Results shown in Fig 1A demonstrate that empty DOPCDOPS liposomes did not stimulate cellular proliferation.

We have screened 25 healthy individuals randomly selected from our blood bank donors. None of them had detectable anti-GpIIb-IIIa autoantibodies in their sera. All PBMC tested proliferated in response to GpIIb-IIIa with stimulation index >3 and Δ cpm >500, with some being marginally positive (3.0) while others were very high (57.0) with an average of 9.9 ± 8.0 (Fig 1B). This variation could be due to their different HLA background. Furthermore, PBMC from several individuals were restested two to four times. The stimulation index obtained was slightly variable and positive in all cases (data not shown).

Inhibition by lysosomotropic agents. To determine whether GpIIb-IIIa was internalized and processed in APC in our assay, we have used two lysosomotropic agents that block the HLA class II presentation pathway. Results shown in Fig 2 indicate that chloroquine and NH₄Cl inhibited T-cell proliferation to DOPC/DOPS liposome-encapsulated GpIIb-IIIa or to control tetanus toxoid antigen when added before but not after APC presentation. Furthermore, these
Fig 1. Proliferation of PBMC in response to Gpllb-IIa. (A) Dose-response analysis of PBMC from three healthy individuals without detectable anti-Gpllb-IIa antibodies in the presence of DOPC/DOPS liposome-encapsulated Gpllb-IIa (●), 0.02% Triton X-100 solubilized Gpllb-IIa (●), 0.02% Triton X-100 buffer without Gpllb-IIa (▲), empty liposomes (♦), or purified fibrinogen (○). At day 7, IL-2 (50 U/mL) was added and incubation continued for 3 more days. Each point represents the average from three healthy individuals of the stimulation index at day 10, using ten replicates per individual. (B) PBMC of 25 healthy individuals without detectable anti-Gpllb-IIa antibodies were incubated for 7 days in the presence of 7.5 μg/mL of liposome-encapsulated Gpllb-IIa. At day 7, IL-2 (50 U/mL) was added and incubation continued for 3 more days. Each point represents the stimulation index at day 10 from a given individual, using ten replicates per individual. One individual with a stimulation index of 57.0 was not included in the figure.

Fig 2. Inhibition of the proliferative response to DOPC/DOPS liposome-encapsulated Gpllb-IIa by lysosomotropic agents. For each assay, PBMCs subjected to different treatments were mixed with an equal number (8 × 10⁴) of cells per well of purified autologous T cells over 7 days. PBMCs were incubated with the antigen for 1 hour at 37°C before γ-irradiation and then mixed with the purified T cells over 7 days (a); (b) As in (a) but with prior treatment of APC with 0.1 mmol/L chloroquine for 30 minutes; (c) as in (a) except that treatment with chloroquine followed incubation with the antigen; (d) as in (a) but with prior treatment of APC with 10 mmol/L NH₄Cl; (e) as in (a) except that treatment with NH₄Cl followed incubation with the antigen; (f) as in (a) but without antigen; (g) as in (a) but with empty liposomes; (h) as in (a) but without APC. (A) With 7.5 μg/mL of liposome-encapsulated Gpllb-IIa or (B) with 1 μg/mL of tetanus toxoid. At day 7, IL-2 (50 U/mL) was added. Data represents the mean cpm count of 10 replicates at day 10 for one individual out of five healthy individuals tested for Gpllb-IIa and out of three healthy individuals for tetanus toxoid. The results were similar for the other individuals.
two agents did not block proliferation due to PHA, a mitogen that does not require APC processing to trigger T-cell proliferation (data not shown). These results indicate that in our assay GpIIb-IIIa was being internalized and processed in APC before presentation to T cells via the HLA class II pathway.

Inhibition by anti-HLA antibodies. Since GpIIb-IIIa requires the HLA class II pathway to activate T cells, it would be expected that HLA-DR, HLA-DP, or HLA-DQ mediated the response to GpIIb-IIIa. Three different blocking MoAbs directed against HLA-DR, HLA-DP, or HLA-DQ class II molecules were used to test this and we found that anti-HLA-DR but not anti-HLA-DP or anti-HLA-DQ significantly inhibited the response to GpIIb-IIIa for three different individuals. The inhibition was 84%, 80%, and 77%, respectively (Fig 3A). T-cell proliferation to control tetanus toxoid antigen was also inhibited by anti-HLA-DR, 90%, 89%, and 85%, respectively (Fig 3B). T-cell proliferation induced by PHA, which does not involve the MHC class II pathway, was not inhibited by any of the anti-HLA class II MoAbs (data not shown). These results indicate that in our assay the response to GpIIb-IIIa was being mediated by the HLA-DR molecules.

Characterization of αβTCR+CD4+CD8− T-cell response to GpIIb-IIIa. We have previously shown that CD4+CD8− T cells activated by DOPC/DOPS liposome-encapsulated GpIIb-IIIa without exogenous IL-2 express IL-2Ra on their cell surface at day 6.14 Here, we have investigated whether this response was mediated by αβTCR+ T cells as would be expected for an antigen presented by HLA-DR class II molecules. We used 3-color FACS analysis with reagents to αβTCR, CD4, and IL-2Ra to follow the appearance and the density of the IL-2Ra on the cell surface of T cells activated by GpIIb-IIIa in comparison with T cells activated by nonspecific varicella zoster (VZ) antigen and to a mitogenic agent (PHA). The majority of T cells activated by GpIIb-IIIa were αβTCR+CD4+CD8− T cells as would be expected for an HLA class II mediated response (Fig 4). The kinetic of T cells activated by self GpIIb-IIIa was similar to the one found with nonspecific VZ (Table 1). Only T cells activated by PHA showed a different kinetic. At day 2, close to 100% of both αβTCR+CD4+ and αβTCR+CD4− T cells express IL-2Ra on their cell surface (Table 1). Furthermore, we have determined that the incubation of empty DOPC/DOPS liposomes with T cells do not induce the expression of IL-2Ra on their cell surface (data not shown).

Interestingly, the IL-2Ra density on the cell surface of the activated T cells was found to be different between the nonspecific VZ antigen and the self GpIIb-IIIa. At day 4 (Fig 4) and day 6 (not shown), the cell surface expression of IL-2Ra was less abundant for αβTCR+CD4+CD8− T cells activated by GpIIb-IIIa than by VZ antigen. In the case of GpIIb-IIIa, the αβTCR+CD4+CD8− T cells could be divided into two populations, a majority of cells with low or intermediate IL-2Ra density and a minority with high density.

DISCUSSION

Intrathymic clonal deletion is viewed as the major mechanism for tolerance induction in class II restricted T cells and should lead to the elimination of autoreactive T cells to circulating antigens.6 Here we have shown that some autoreactive T cells to GpIIb-IIIa, a membrane antigen expressed as early as at 16 weeks of intrauterine life on bone marrow-derived cells and on the thymus, are not deleted and are present in the periphery of healthy individuals. In order to induce the proliferation of these autoreactive αβTCR+CD4+CD8− T cells in vitro, GpIIb-IIIa must be internalized and processed by APC and then presented by HLA-DR class II molecules in the presence of exogenous IL-2.

It is surprising to find autoreactive T cells directed to GpIIb-IIIa in healthy individuals, since Semple and Freedman12 and Ware and Howard,13 using an in vitro assay with whole platelets, had found the presence of autoreactive T cells in patients with AT but not in healthy individuals. This could be due to the fact that they did not use exogenous IL-2 to stimulate proliferation12 or did not carry it out in the same manner.13 Furthermore, they used whole platelets instead of purified GpIIb-IIIa. When we used whole platelets in our assay, we did not see proliferation either. The autore-
active T cells to GpIlb-IIIa present in healthy individuals seem to be regulated by a mechanism of peripheral tolerance with characteristics similar to but distinct from clonal anergy. It appears that the addition of exogenous IL-2 with GpIlb-IIIa can break this tolerance, at least in vitro.

Table 1. Kinetics of αβ TCRαβCD4+ T-Cell Activation Based on IL-2R-α Expression by FACS

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>GpIlb-IIIa</td>
<td>11.0%</td>
<td>5.7%</td>
<td>64.0%</td>
</tr>
<tr>
<td>VZ</td>
<td>5.2%</td>
<td>13.1%</td>
<td>12.4%</td>
</tr>
<tr>
<td>PHA</td>
<td>99.0%</td>
<td>98.5%</td>
<td>87.6%</td>
</tr>
</tbody>
</table>

PBMG, 5 × 10⁶, from two healthy individuals (A and B) were labelled after 2, 4, and 6 days in the presence of 7.5 μg/mL of liposome-encapsulated GpIlb-IIIa, 10 μg/mL of VZ antigen, and 1 μg/mL of PHA with biotinylated MoAbs to CD4 with streptavidin Cy-chrome, αβ TCR followed by FITC-conjugated goat antioimmunoglobulin lgs and PE-conjugated IL-2R-α. The results are expressed as the proportion of αβ TCRαβCD4+ T cells positive for IL-2 receptor α over the total αβ TCRαβCD4+ T-cell population (day 0 < 1%).

Abbreviation: ND, not done.

The presence of reactive T cells to a blood-borne self antigen in healthy individuals has also been observed recently by Barker and Elson. They have shown that healthy individuals carried, in their peripheral blood, T cells reacting to the Rhesus self antigen present on red blood cells. However, in their case, these cells proliferated and produced IL-2 when put in contact with Rhesus synthetic peptides or with whole red blood cells. Thus, these T cells did not appear to be regulated by peripheral tolerance as the GpIlb-IIIa autoreactive T cells, but rather behaved as if they had not been previously in contact with the antigen.

GpIlb-IIIa is known to contain several polymorphisms that are targets for antibodies leading to neonatal thrombocytopenia or to posttransfusion purpura (HPA system). Previously, we have shown that polymorphism was not the reason for T-cell stimulation because we found no difference between PBMC originating from alloimmunocompatible individuals for GpIlb-IIIa (HPA-1 and HPA-3 system) and from unmatched PBMC in their capacity to proliferate to DOPC/DOPS liposome-encapsulated GpIlb-IIIa and exogenous IL-2. Usually, naive or nonexperimented T cells secrete IL-2 and proliferate strongly without the need for exogenous IL-2 when they respond to a foreign antigen, to a neoantigen, or to an alloanitgen. The results presented here indicate that the response obtained with purified GpIlb-
GpIIb-IIIa molecules on their cell surface, which represent approximately 1% to 2% of the total platelet proteins both for fetuses and adults. Furthermore, it has been shown that GpIIb-IIIa is also present on the membrane of the epithelial cells of the thymic stroma as early as the 16th week of gestation. Several results suggest that self antigens present in the thymus at such time and in such amount should induce clonal deletion. Intrathymic injection, transplantation, and transgenic expression in the thymus of self membrane antigens in animals have been shown to induce clonal deletion of the T cells reactive to these antigens.

Another possibility to explain the presence of some autoreactive T cells to GpIIb-IIIa could be that the interaction between some thymic HLA/GpIIb-IIIa/TCR is one of low affinity. With regards to TCR affinity, it is generally assumed, though not formally proven, that high affinity interactions lead to clonal deletion, while T cells with a TCR of low affinity will survive but are rendered anergic in the periphery. Thus, one could envisage that the autoreactive T cells with high affinity to GpIIb-IIIa are deleted during the process of thymic education while autoreactive T cells with low affinity to GpIIb-IIIa escape thymic deletion. The observation that the majority of the autoreactive T cells analyzed express low or intermediate IL-2Ra density in response to GpIIb-IIIa is in agreement with this possibility.

In conclusion, the results presented here indicate that autoreactive T cells to self GpIIb-IIIa, present on bone marrow-derived cells as well as on the membrane of thymic epithelial cells, are present in most, if not all, healthy individuals. This strongly suggests that these T cells escaped thymic deletion and are tolerized in the periphery to prevent an autoimmune reaction against GpIIb-IIIa.

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Presence in peripheral blood of healthy individuals of autoreactive T cells to a membrane antigen present on bone marrow-derived cells