Soluble HLA-G protein secreted by allo-specific CD4$^+$ T cells suppresses the allo-proliferative response: A CD4$^+$ T cell regulatory mechanism

Nermine Lila*†, Nathalie Rouas-Freiss*, Jean Dausset‡, Alain Carpentier†§, and Edgardo D. Carosella*¶

*Service de Recherches en Hémato-Immunologie, Commissariat à l'Energie Atomique, Direction des Sciences du Vivant, Département de Recherche Médicale, Hôpital Saint-Louis, 1 Avenue Claude-Vellefaux, 75010 Paris, France; †Laboratory for the Study of Cardiac Grafts and Prostheses, University Pierre et Marie Curie, Hôpital Broussais, 96 Rue Didot, 75014 Paris, France; ‡Centre d'Étude du Polymorphisme Humain, Fondation Jean Dausset, 27 Rue Juliette Dodou, 75010 Paris, France; and §Department of Cardiovascular Surgery and Organ Transplantation, Georges Pompidou Hospital, 75015 Paris, France

Contributed by Jean Dausset, August 2, 2001

We recently reported that the nonclassical HLA class I molecule HLA-G was expressed in the endomyocardial biopsies and sera of 16% of heart transplant patients studied. The aim of the present report is to identify cells that may be responsible for HLA-G protein expression during the allogeneic reaction. Carrying out mixed lymphocyte cultures in which the responder cell population was depleted either in CD4$^+$ or CD8$^+$ T cells, we found that soluble HLA-G5 protein but not the membrane-bound HLA-G isoform was secreted by allo-specific CD4$^+$ T cells from the responder population, which suppressed the allogeneic proliferative T cell response. This inhibition may be reversed by adding the anti-HLA-G 87G antibody to a mixed lymphocyte culture. That may indicate a previously uncharacterized regulatory mechanism of CD4$^+$ T cell proliferative response.

The nonclassical HLA class I antigen HLA-G, physiologically expressed on extravillous cytotrophoblast at the feto-maternal interface during pregnancy (1, 2), plays an important role in protecting the semiallogeneic fetus from maternal immune attack and subsequent rejection (3). A particular characteristic of HLA-G is alternative splicing of its primary transcript, resulting in four membrane-bound proteins, HLA-G1, -G2, -G3, and -G4, as well as in three soluble isoforms, HLA-G5, -G6 and -G7 (4–7). Both the HLA-G5 and -G6 soluble isoforms are produced by alternative transcripts that lack exons for encoding both the transmembrane and cytoplasmic domains. These soluble proteins have been detected in the amniotic fluid and serum of pregnant women (8). Interestingly, HLA-G expression has also been observed in thymic epithelial cells (9), cytokine-activated monocytes (10, 11), macrophages/dendritic cells during viral infections (12), and in various tumors (13–15). Soluble HLA-G has been demonstrated to induce apoptosis of activated CD8$^+$ T cells (16) and to modulate the natural killer cell (17) and allo-cytotoxic T lymphocyte responses (18), whereas membrane-bound HLA-G proteins have been shown to inhibit both natural killer cell and T cell-mediated cytolysis (19), to suppress proliferation of allo-specific CD4$^+$ T lymphocytes (20, 21), and to induce a Th2 cytokine profile (22, 23). HLA-G interacts with inhibitory receptors such as ILT-2, ILT-4, p49, and KIR2DL4 expressed on natural killer cells, T cells, monocytes, and dendritic and/or B cells (24–28).

We have demonstrated recently that HLA-G may be expressed in biopsies and sera of patients who have undergone heart transplantation and may be associated with better graft acceptance (29). The aim of the present study in this regard is to identify cells responsible for HLA-G expression during the *in vitro* allogeneic reaction. For this purpose, we carried out mixed lymphocyte cultures by using peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteer donors that express distinct MHC class I and II molecules, thereby mimicking the allogeneic reaction observed after organ transplantation between histo-incompatible individuals. Based on the previous work of Carosella *et al.* showing that allo-sensitized T cells indeed produce soluble factors that are able to suppress allogeneic proliferation (30, 31), we investigated whether soluble HLA-G proteins were secreted during the mixed lymphocyte reaction (MLR), thereby affecting the allogeneic immune response.

Materials and Methods

Cell Preparation and Culture. PBMCs obtained from heparinized whole blood of four healthy volunteer adult donors were obtained by density-gradient centrifugation over Ficoll-Histopaque (density 1.077 g/ml) (Amersham Pharmacia). The PBMCs were washed twice with RPMI medium 1640 and suspended in culture medium consisting of RPMI medium 1640 supplemented with Fungizone (Sigma), 1 μg/ml gentamicin, L-glutamine (GIBCO/BRL), and 10% heat-inactivated human serum AB at concentrations of 1×10^6 responder cells/ml and 1×10^6 stimulator cells per ml. All stimulator cells were prepared by γ irradiation at 25 grays. Responder cells were depleted in either CD4$^+$ or CD8$^+$ T cells in specific experiments as follows: first, CD4$^+$ or CD8$^+$ T cell depletion was accomplished by using anti-CD4- or anti-CD8-coated Dynabeads (Dynal, Oslo, Norway). Coated dynabeads then were mixed with PBMCs of the responder population at a 4:1 ratio of beads to target CD4$^+$ or CD8$^+$ T cells. After incubation at 4°C for 1 h, the target cells rosetted with Dynabeads were depleted by using Dynal magnetic particle concentrators.

MLR. Nine different responder/stimulator cell combinations were established, and three autologous combinations were used as controls (responder and stimulator cells from the same donor) (Table 1). In 96 U-bottomed plates, 5×10^4 responder cells (corresponding either to total cells, CD4$^+$-depleted, or CD8$^+$-depleted) were cultured with 5×10^4 irradiated stimulator cells and plated in a final volume of 150 μl per well. All samples were run in triplicate. Cultures were incubated at 37°C in a humidified 5% CO_2 air atmosphere for 5 days and then pulsed with thymidine [1 μCi per well (1 Ci = 37 GBq), Amersham Pharmacia]. Cells were harvested 18h later on filter mats, and thymidine incorporation into DNA was quantified by using a beta counter (Wallac 1450, Amersham Pharmacia). The anti-HLA-G5 87G mAb antibody was added in four combinations on the first and third days of mixed lymphocyte culture.

Concomitant with the above-mentioned MLR in 96-well plates, we incubated these various MLR combinations in flasks by using 5×10^6 responder cells and 5×10^6 stimulator cells (1×10^6/ml) at 37°C in a humidified 5% CO_2 air atmosphere for 10 days. Restimulation then was carried out under the same conditions, and the resulting secondary immune response was

Abbreviations: PBMC, peripheral blood mononuclear cell; MLR, mixed lymphocyte reaction.

¶To whom reprint requests should be addressed. E-mail: carosella@dsvidf.cea.fr.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Table 1. Combinations of mixed lymphocytes cell cultures tested

Stimulator	Responder: Undepleted I	I ($CD4^-CD8^+$)	I ($CD4^+CD8^-$)
1	I + 1	I ($CD4^-CD8^+$) + 1	I ($CD4^+CD8^-$) + 1
2	I + 2	I ($CD4^-CD8^+$) + 2	I ($CD4^+CD8^-$) + 2
3	I + 3	I ($CD4^-CD8^+$) + 3	I ($CD4^+CD8^-$) + 3
4	I + 4	I ($CD4^-CD8^+$) + 4	I ($CD4^+CD8^-$) + 4
Stimulator	**Responder: Undepleted II**	**II ($CD4^-CD8^+$)**	**II ($CD4^+CD8^-$)**
1	II + 1	II ($CD4^-CD8^+$) + 1	II ($CD4^+CD8^-$) + 1
2	II + 2	II ($CD4^-CD8^+$) + 2	II ($CD4^+CD8^-$) + 2
3	II + 3	II ($CD4^-CD8^+$) + 3	II ($CD4^+CD8^-$) + 3
4	II + 4	II ($CD4^-CD8^+$) + 4	II ($CD4^+CD8^-$) + 4
Stimulator	**Responder: Undepleted III**	**III ($CD4^-CD8^+$)**	**III ($CD4^+CD8^-$)**
1	III + 1	III ($CD4^-CD8^+$) + 1	III ($CD4^+CD8^-$) + 1
2	III + 2	III ($CD4^-CD8^+$) + 2	III ($CD4^+CD8^-$) + 2
3	III + 3	III ($CD4^-CD8^+$) + 3	III ($CD4^+CD8^-$) + 3
4	III + 4	III ($CD4^-CD8^+$) + 4	III ($CD4^+CD8^-$) + 4

(Top) Responder cells are from the donor I, and stimulator cells are from either the donor 1 (autologous control) or from donors 2, 3, and 4 (allogeneic combinations). (Middle) Responder cells are from donor II, and stimulator cells are from either donor 2 (autologous control) or the donors 1, 3, and 4 (allogeneic combinations). (Bottom) Responder cells are from the donor III, and stimulator cells are from either donor 3 (autologous control) or donors 1, 2, and 4 (allogeneic combinations).

analyzed on days 12 and 15. After 10 days of MLR, the cells were counted, and their viability was evaluated by trypan blue exclusion. Cells were then studied by cytofluorometry (FACScalibur) and immunocytochemistry analysis. Culture supernatants were also collected to detect expression of soluble HLA-G isoforms by immunoprecipitation followed by Western blot analysis.

mAbs and Flow Cytometry Analysis. By using flow cytometry analysis, we determined the percentage of each cell population and checked $CD4^+$ and $CD8^+$ T cell depletion on days 0 and 10 of MLR. Cells (1×10^4) were washed in PBS and 2% FCS and then incubated with 50% human normal serum for 30 min. The cells were incubated with the following antibodies: CD4, phycoerythrin (CD4PE); CD8, phycoerythrin (CD8PE); and murine isotype control conjugated with phycoerythrin (Immunotech, Marseille, France) for 30 min at 4°C. After 10 days of MLR, responder T cells were analyzed for HLA-G surface expression by using the mAbs 87G IgG2a anti-HLA-G1 and -G5 (kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Institute, Seattle, WA) and 4H84 IgG1, which recognizes the α1 domain (pan-HLA-G) (kindly provided by M. McMaster, University of California, San Francisco, CA). An isotype-matched antibody was used as the control. The cells were incubated for 30 min at 4°C, washed with PBS, and incubated with goat anti-mouse antibody coupled with FITC (Sigma) for 30 min.

Apoptosis Detection. For apoptosis detection, cells were labeled with Annexin V FITC (Immunotech), washed with PBS, and analyzed by using a FACScan flow cytometer. PBMCs were treated with staurosporine (Roche Molecular Biochemicals) as a positive control for apoptosis. The data are expressed as the percentage of cells positively stained by the indicated reagent.

Immunoprecipitation and Western Blot Analysis. The M8 melanoma cell line was transfected with a full-length HLA-G5 cDNA subcloned in a vector pcDNA (Invitrogen) as described previously (7, 20). For Western blot analysis, the M8-HLA-G5 and M8-HLA-G1 transfectants were used as positive controls, and M8-pcDNA transfected with the vector alone was used as the negative control. During MLR, culture supernatants were collected on days 3, 6, and 10 of the primary reaction and on days 12 and 15 after restimulation, then concentrated 10×, by using a centrifugal filter device (Millipore) and stored at −80°C until used for immunoprecipitation and Western blot analysis.

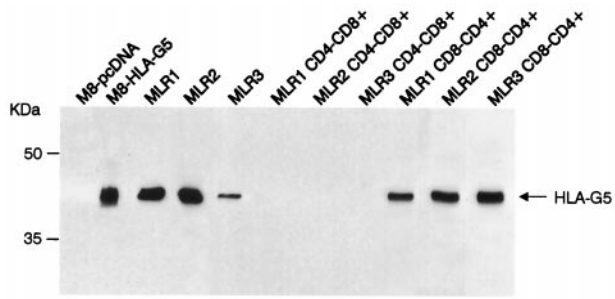

Fig. 1. Immunoprecipitation followed by Western blot analysis of cell culture supernatants after 10 days of MLR revealed the presence of soluble HLA-G5 in three MLR combinations (MLR1, MLR2, and MLR3). Immunoprecipitation and Western blot analysis of soluble HLA-G were carried out by using the PAG5-6 Ab and 4H84 mAb, respectively. In these HLA-G5-positive combinations, when the responder cells were $CD4^+$-depleted (MLR1 $CD4^-CD8^+$, MLR2 $CD4^-CD8^+$, and MLR3 $CD4^-CD8^+$), HLA-G5 could not be detected. In contrast, when responder cells were $CD8^+$-depleted (MLR1 $CD8^-CD4^+$, MLR2 $CD8^-CD4^+$, and MLR3 $CD8^-CD4^+$), HLA-G5 could be observed. M8-HLA-G5 and M8-pcDNA transfectant cells were used as positive and negative controls, respectively.

Immunoprecipitation of the MLR culture supernatant was accomplished by using PAG5-6, a rabbit polyclonal antibody specific for soluble HLA-G5 and HLA-G6 (7). Cell culture supernatant was incubated overnight at 4°C with PAG5-6. Protein A-Sepharose beads were added and incubated for 1 h at 4°C. Beads bearing the immune complexes were washed and incubated for 5 min at 95°C with 1% SDS sample buffer and 150 mM β-mercaptoethanol. Immunoprecipitation was followed by Western Blot analysis as described below.

Concomitantly, HLA-G proteins were studied by Western blot analysis in cell lysates obtained after the primary and secondary immune responses. For this purpose, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40) containing protease inhibitors (Roche Molecular Biochemicals) for 40 min at 4°C. Cell fragments were removed by centrifugation at 14,000 rpm for 30 min at 4°C, and supernatants were used for Western blot analysis as follows: 25-μl aliquots of total protein from the indicated samples were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond, Amersham Pharmacia). After blocking in 5% nonfat

Table 2. Presentation of the mixed lymphocytes cell cultures tested and the corresponding HLA-G production

Stimulator	Responder: I HLA-G*	II HLA-G	III HLA-G
1	−	−	−
2	+	−	+
3	−	+	−
4	−	−	−

The responder and stimulator cells are listed according to their respective donors.

*The data correspond to the product of soluble HLA-G during the MLR indicated in the table.

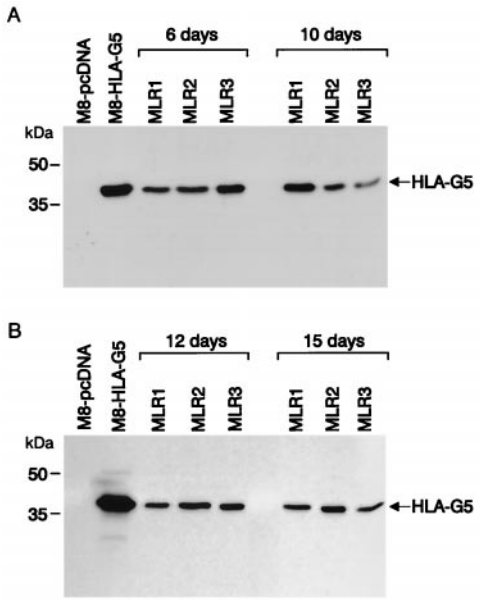

Fig. 2. Detection of HLA-G5 by immunoprecipitation followed by Western blot analysis of cell culture supernatants on various days after MLR. Stimulator cells were used against total peripheral blood mononuclear responder cells in the three HLA-G-positive combinations (MLR1, MLR2, and MLR3). (*A*) Days 6 and 10 of primary MLR. (*B*) Days 12 and 15 of secondary MLR. M8-HLA-G5 transfectant cells were used as the positive control, and M8-pcDNA was used as the negative control.

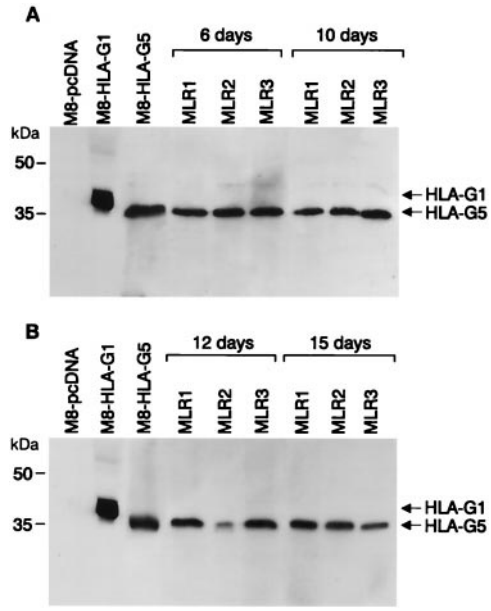

Fig. 4. Western blot analysis of responder cell lysates after MLR. Stimulator cells were used against total peripheral blood mononuclear responder cells in the three HLA-G-positive combinations (MLR1, MLR2, and MLR3). (*A*) Days 6 and 10 of primary MLR. (*B*) Days 12 and 15 of secondary MLR. M8-HLA-G5 and M8-HLA-G1 transfectant cells were used as positive controls, and M8-pcDNA was used as the negative control.

milk in PBS/0.2% Tween, the membrane was incubated with the 4H84 mAb overnight at 4°C and washed in PBS/0.2% Tween. The membrane was then incubated for 30 min at room temperature with anti-mouse peroxidase conjugate reagent (Amersham Pharmacia). After washing, the staining reaction was carried out by using ECL Western blotting detection (Amersham Pharmacia), after which the membrane was exposed to Kodak film.

Immunocytochemical Analysis. For immunocytochemical studies, cells were fixed for 10 min in cold acetone, dehydrated, and permeabilized or not with saponin in PBS. Staining procedures

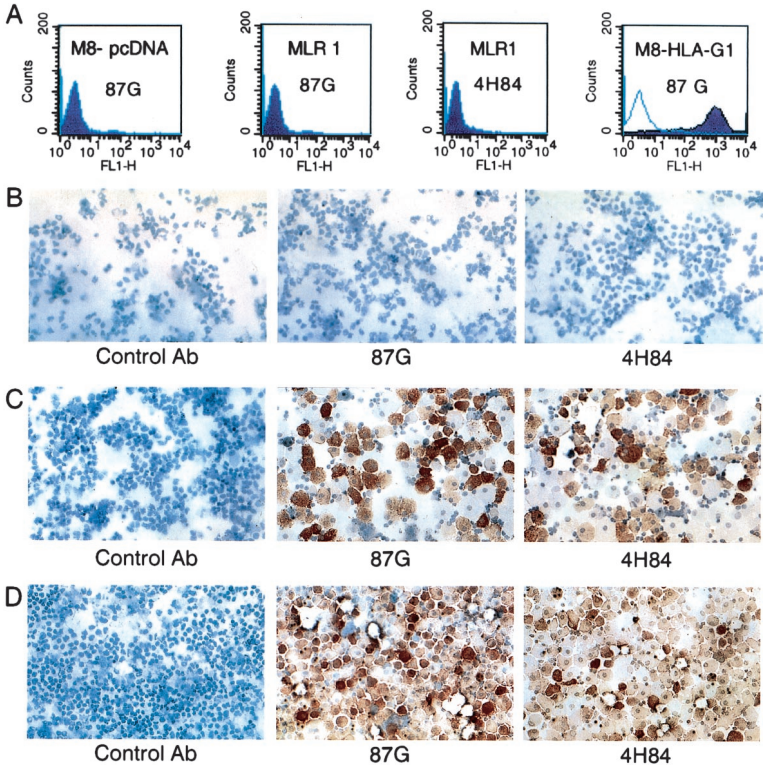

Fig. 3. Flow cytometry and immunocytochemical analysis after 10 days of MLR by using the 87G and 4H84 anti-HLA-G mAbs. (*A*) Flow cytometry profiles were all negative with anti-HLA-G mAbs, whereas HLA-G1-transfected cells were positively stained with 87G. (*B*) Immunocytochemical analysis was carried out without permeabilization. In this HLA-G-positive combination, the PBMCs were negatively stained; after permeabilization, the responder cells correspond to all of the PBMCs (*C*) and $CD8^+$-depleted PBMCs (*D*) were positively stained.

Table 3. Tritiated thymidine incorporation after 6 days of mixed lymphocyte reaction between stimulator and responder cells corresponding to either total PBMCs, CD4$^+$-depleted PBMCs, or CD8$^+$-depleted PBMCs

	MLR	
Responder cells	Autologous reaction	Allogeneic test
Total PBMCs	8,794 ± 4,127*	47,697 ± 14,880
CD4$^+$-depleted T cell (CD4$^-$CD8$^+$)	8,418 ± 1,255	18,617 ± 8,989†
CD8$^+$-depleted T cell (CD4$^+$CD8$^-$)	6,884 ± 3,923	33,214 ± 14,431‡

*cpm $\bar{X}$ ± SEM.
† vs. ‡, $P < 0.018$.

were processed at room temperature by using the Dako Envision system (Dako). Endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 5 min. After washing, the cells were incubated for 20 min in 50% human normal serum to eliminate nonspecific binding. Samples were incubated with the following primary antibodies for 30 min: W6/32 IgG2a pan-HLA class I; 87G; 4H84; and control antibody. The samples were then incubated with a secondary conjugated goat anti-mouse antibody coupled with peroxidase (Dako) for 30 min. After a 10-min incubation with substrate, the cells were counterstained with hematoxylin dye and mounted with anti-mounting medium (Dako).

Statistics. Data are presented as means ± SEM. Student's *t* test was used and a $P < 0.05$ was taken to be significant.

Results

Detection of Soluble HLA-G5 in Three Combinations After MLR. First, we investigated whether soluble HLA-G could be expressed spontaneously during mixed lymphocyte culture. For this purpose, we carried out immunoprecipitation by using PAG5-6, a rabbit polyclonal antibody specific for the soluble HLA-G5 and G6 proteins, followed by Western blot analysis by using the 4H84 mAb, specific for the HLA-G α1 domain. We analyzed the presence of soluble HLA-G in both cell lysates and supernatants obtained from various combinations of primary MLR on days 3, 6, and 10 and of secondary MLR on days 12 and 15.

As shown in Fig. 1, on day 10 of MLR, the results revealed a band at 37 kDa in three of nine MLR combinations corresponding to the detection of soluble HLA-G5 in MLR supernatants (Table 2). In a second step, we attempted to determine which cell type was responsible for such HLA-G production. We therefore carried out depletion experiments in which CD8$^+$ or CD4$^+$ T cells from the responder population were eliminated from the MLR. In all three HLA-G-positive combinations in which responder cells were depleted among the CD8$^+$ T cell population, soluble HLA-G5 was still detected. In contrast, no HLA-G production was observed when responder cells were depleted in CD4$^+$ T cells. Transfected M8-HLA-G5 cells were used as the HLA-G5-positive control (Fig. 1). We then attempted to determine at which point HLA-G5 could be detected in the MLR culture supernatant. For this, the three positive combinations were studied similarly on days 3, 6, and 10 of primary MLR and on days 12 and 15 of the secondary MLR. The results showed that production of soluble HLA-G5 was absent on day 3 (data not shown), whereas it was detected on days 6 and 10 of the primary MLR and on days 12 and 15 of the secondary MLR (Fig. 2).

Analysis of HLA-G Expression by Flow Cytometry and Immunocytochemistry. To investigate further HLA-G expression during MLR, cells were collected on day 10 of the primary response and analyzed by both flow cytometry and immunocytochemistry. To detect membrane-bound HLA-G molecules, flow cytometry analysis experiments were carried out on the responder cell population of the three HLA-G-positive combinations after 10 days of MLR. The 87G (specific for HLA-G1 and -G5) and the 4H84 (specific for the HLA-G α1 domain) mAbs were used. The results showed that flow cytometry profiles were all negative with both anti-HLA-G mAbs, whereas the HLA-G1-transfected cells were positively stained (Fig. 3). These results demonstrated no cell surface HLA-G expression after the allogeneic response. Similar experiments were conducted on HLA-G-negative combinations, and no HLA-G expression was detected (data not shown).

We then carried out immunocytochemistry experiments to detect intracytoplasmic HLA-G expression. The results showed that HLA-G expression was only observed in the three HLA-G-positive combinations described above in which cells of the same sample were positively stained by either the 87G or 4H84 anti-HLA-G mAbs. Fig. 3 presents the results obtained with one HLA-G-positive combination taken as a representative example, showing that (*i*) when all the PBMCs were used as responders, 60% of the cells were positively stained by one or the other of the two anti-HLA-G mAbs, and (*ii*) when CD8$^+$-depleted PBMCs were used as responders, 90% of the cells were positively stained by one or the other of the two anti-HLA-G mAbs. When similar experiments were carried out without permeabilizing the cells, no staining was detected when the anti-HLA-G mAbs were used (Fig. 3). These results confirmed that no HLA-G molecules were expressed at the cell surface.

Analysis of HLA-G Isoform Expression by Western Blot. Having demonstrated that HLA-G5 is secreted in supernatants obtained from particular MLR combinations, whereas no cell surface HLA-G proteins could be observed in them, we then raised the question of whether HLA-G isoforms could be detected intracellularly in these same three HLA-G-positive combinations. For this purpose, we carried out Western blot analysis by using the 4H84 mAb on cell lysates from the three HLA-G-positive combinations on days 6 and 10 of primary MLR and on days 12 and 15 of secondary MLR. M8-HLA-G1- and M8-HLA-G5-transfected cells were used as positive controls for the detection of the HLA-G1 (39 kDa) and HLA-G5 (37 kDa) proteins, respectively. Interestingly, the results showed that only a band at

Table 4. Tritiated thymidine incorporation in HLA-G-positive and -negative combinations after 6 days of mixed lymphocyte reaction between stimulator cells and responder cells corresponding to either total PBMCs, CD4$^+$-depleted PBMCs, or CD8$^+$-depleted PBMCs

	MLR		
Responder cells	HLA-G5-positive combinations ($n = 3$)	HLA-G5-negative combinations ($n = 6$)	$P <$
Total PBMCs	50,652 ± 19,188*	46,215 ± 14,112	0.702
CD4$^+$-depleted T cell (CD4$^-$CD8$^+$)	16,020 ± 6,867	19,915 ± 10,215	0.575
CD8$^+$-depleted T cell (CD4$^+$CD8$^-$)	18,217 ± 7,480	40,712 ± 10,412	0.013*
CD8$^+$-depleted T cell (CD4$^+$CD8$^-$) + 87G mAb	79,298 ± 2,553	44,024 ± 3,234	0.006*

*cpm $\bar{X}$ ± SEM.

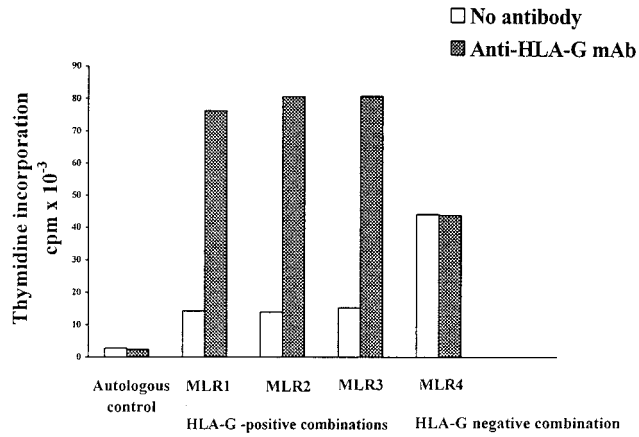

Fig. 5. Effect of HLA-G5 production on T cell proliferation when (*i*) the responder cells were $CD8^+$-depleted ($CD4^+$-enriched) and (*ii*) the anti-HLA-G5 mAb was used in HLA-G-positive and -negative combinations. The data are expressed as the mean of thymidine incorporation (cpm) in triplicate wells.

37 kDa, corresponding to the HLA-G5-soluble isoform, could be observed (Fig. 4). It is noteworthy that this result constitutes the first observation of the absence of expression of the full-length HLA-G1 membrane-bound protein at the same time that another HLA-G form is expressed in it (i.e., HLA-G5).

Analysis of the T Cell Alloproliferative Response After 6 Days of MLR. To evaluate the effects of the HLA-G5 protein secreted during MLR, we compared the T cell alloproliferative response obtained in positive and negative HLA-G combinations. When the responder cells were $CD8^+$-depleted PBMCs or $CD4^+$-depleted PBMCs, the results showed (*i*) very low levels of proliferation in the autologous control wells and (*ii*) a strong proliferative response in all allogeneic combinations tested (Table 3). Table 4 compares the allo-proliferative responses of the three HLA-G-positive and the six HLA-G-negative combinations. In the three HLA-G-positive combinations, when the responder cells were $CD4^+$-depleted ($CD8^+$-enriched), there was no significant difference in the allo-proliferative response between HLA-G-positive and -negative combinations. In contrast, when the responder cells were $CD8^+$-depleted ($CD4^+$-enriched), the allo-proliferative response was decreased significantly in HLA-G-positive combinations ($P < 0.013$). This suppression of the allo-proliferative response may be caused by the secretion of HLA-G5 in the HLA-G-positive combinations. This hypothesis was confirmed by using the anti-HLA-G5 87G mAb on the first and third days of MLR, which restored T cell allo-proliferation in the HLA-G-positive combinations, whereas no effect was obtained in the HLA-G-negative combinations (Table 4 and Fig. 5). These results show that HLA-G5 secreted by allo-specific $CD4^+$ T cells is involved in the suppression of $CD4^+$ T cells. The proportion of $CD4^+$ and $CD8^+$ T cells in PBMCs from all tested donors was of 64.5 ± 8% for $CD4^+$ T cells and 20.3 ± 0.3% for $CD8^+$ T cells. Depletion of either $CD4^+$ or $CD8^+$ T cells was successful, because 0% of both $CD4^+$ and $CD8^+$ T cells were detected by flow cytometry after depletion. After 10 days of MLR, we investigated whether the percentage of T cells in HLA-G-positive and -negative combinations was modified. Flow cytometry analysis by using anti-CD4 and anti-CD8 mAbs showed no difference between these combinations (Table 5).

Apoptosis Detection. Soluble HLA-G5 has been demonstrated previously to induce apoptosis of activated $CD8^+$ T cells (17). For this purpose, we studied whether apoptosis could be detected in HLA-G-positive combinations. The results showed that after 10 days of MLR, apoptosis was detected in all MLR combinations: 62 ± 14% in total cells, 72.8 ± 7.6% in $CD4^+$-depleted responder cells, and 64.32 ± 9.1% in $CD8^+$-depleted responder cells. No apoptosis was detected in the autologous controls. No significant difference was observed between HLA-G-positive and HLA-G-negative combinations (Table 6).

Discussion

HLA-G expression after heart transplantation has been proposed as a possible mechanism for the induction of graft tolerance (29). Indeed, HLA-G expression has been found to be associated with a decreased incidence of acute rejection episodes and an absence of chronic rejection in 16% of heart transplant patients, compared with heart transplant patients who did not express HLA-G.

The aim of the present study was to identify the cell population responsible for HLA-G expression during the *in vitro* allogeneic reaction. Because heart transplantation is always conducted in histo-incompatible situations, we carried out mixed lymphocyte cultures by using PBMCs from histo-incompatible healthy adult donors. We first analyzed both membrane-bound and soluble HLA-G expression by means of flow cytometry and HLA-G-specific immunoprecipitation followed by Western blot. Our study involved collecting cells and supernatants at various times after the primary and secondary allogeneic reactions. The results revealed that no HLA-G expression could be detected in the autologous combinations, whereas the production of soluble HLA-G5 protein was detected in three of nine allogeneic combinations. Until day 3, HLA-G5 could not be detected in MLR culture supernatants. In contrast, HLA-G5 was detected on days 6 and 10 of the primary immune response and on days 12 and 15 of the secondary immune response. These results suggest that activation of both HLA-G gene transcription and protein expression is a process that occurs subsequent to the early allo-specific T cell response or the secretion of cytokines after allogeneic stimulation. Experiments in which $CD4^+$ or $CD8^+$ T cells were depleted enabled us to identify a cell population implicated in HLA-G5 production. Indeed, we demonstrate that $CD4^+$ T cells from the responder cell population produce soluble HLA-G5 after allogeneic activation. Interestingly, we have only detected the presence of the soluble HLA-G5 isoform and not the membrane-bound HLA-G forms. This may be because of a specific regulation process in the alternative splicing of the primary HLA-G transcript, which could favor selection of the soluble G5 isoform in activated $CD4^+$ T cells. In addition to allo-specific $CD4^+$ T cells, the HLA-G5 molecule is secreted during

Table 5. Percentage of $CD4^+$ and $CD8^+$ T cell populations by flow cytometry analysis after 10 days of MLR between stimulator and responder cells corresponding either to total PBMCs, $CD4^+$-depleted PBMCs, or $CD8^+$-depleted PBMCs

	HLA-G5 positive combinations ($n = 3$)		HLA-G5 negative combinations ($n = 6$)	
Responder cells	% $CD4^+$	% $CD8^+$	% $CD4^+$	% $CD8^+$
Total PBMCs	57.37 ± 9.6	27.32 ± 5.6	57.03 ± 7.7	27.09 ± 6.3
$CD4^+$-depleted T cell	0	63.72 ± 27.6	0	69.51 ± 22.9
$CD8^+$-depleted T cell	82.32 ± 25.1	0	80.49 ± 19.3	0

Table 6. Percentage of apoptosis of the indicated responder cells after 10 days of MLR in both HLA-G-positive and -negative combinations

	Detection of cell apoptosis, %		
Responder cells	HLA-G5-positive combinations ($n = 3$)	HLA-G5-negative combinations ($n = 6$)	$P<$
Total PBMCs	64.9 ± 15.8	60.76 ± 14.7	0.706
$CD4^+$-depleted T cells	77 ± 10.6	70.8 ± 5.6	0.274
$CD8^+$-depleted T cells	71.9 ± 3.3	60.5 ± 8.6	0.068

pregnancy by the cytotrophoblast and activated placental macrophages (32) and is also detected in the serum and amniotic fluid of pregnant women (8). In this regard, we hypothesize that an allogeneic reaction toward paternal antigens during pregnancy is a possible mechanism responsible for the production of HLA-G5 by maternal $CD4^+$ T cells. Although T cells play a major role in graft rejection, we show here that they can also naturally produce soluble HLA-G after the allogeneic reaction. In our study, after $CD8^+$ depletion, leading to $CD4^+$ enrichment, the allogeneic proliferative response was reduced significantly in the HLA-G-positive combinations, compared with the HLA-G-negative combinations ($P < 0.013$). We suggest that an optimal quantity of secreted HLA-G proteins is required to diminish the allogeneic reaction. Because soluble HLA-G has been demonstrated previously to induce apoptosis of $CD8^+$-activated T cells (16), we examined whether this process occurred in our experiments. No significant difference in apoptosis was detected between HLA-G-positive and -negative combinations.

In previous studies, membrane-bound HLA-G protein has been shown to inhibit T cell allo-proliferation (20), and the soluble HLA-G protein produced as a fusion protein in *E. coli* by using the glutathione *S*-transferase system was able to inhibit the natural killer cell activity (17). There was a direct correlation between the HLA-G concentration used and the inhibition obtained (17).

Significantly, in our study the inhibitory effect of HLA-G on the allo-proliferative response could be observed only when the responder cell population was enriched in allo-$CD4^+$ T cells. This result is supported by the demonstration that 87G, an antibody that is specific for HLA-G5, reverses such inhibition. In view of the preceding, we propose that soluble HLA-G5 protein expression could control the allogeneic reaction by limiting T cell allorejection. It is of note that in previous studies, Carosella *et al.* have shown that allo-sensitized T cells produce two types of soluble factors able to suppress allogeneic proliferation: one is restricted to the responder cell population, and the other is unrestricted (30, 31). The present study recalls and confirms this prior experiment, possibly identifying HLA-G as one of the soluble factors restricted to $CD4^+$ T cells from among the responder population. Our previous observation of HLA-G expression in 16% of heart-graft patients in a study is in agreement with the present results. Indeed, only three of the allo-combinations were capable of producing soluble HLA-G, probably because of the fact that HLA-G-positive individuals carry specific HLA-G alleles associated with high HLA-G production (33). However, it is of note that the same responder cells did not produce HLA-G5 when they were in contact with all of the allogeneic stimulator cells tested (i.e., responder cells from donor 1 produce HLA-G5 only with stimulator cells from donor 2 but not from donors 3 and 4). This leads us to the conclusion that HLA-G5 production not only depends on the responder cells but also on specific parameters from particular responder/stimulator combinations. Finally, other factors such as cytokines may play important roles in inducing HLA-G expression. It has been demonstrated that IL10, INF-β, and INF-γ up-regulate HLA-G expression (10, 34–36). We cannot exclude that in particular responder/stimulator combinations, secreted cytokines induce HLA-G expression, both of which may control the allogeneic reaction. Based on the present study, soluble HLA-G5 detected in the sera of heart-transplanted patients may be produced by recipients' allo-specific $CD4^+$ T cells. In conclusion, based on our previous results showing that HLA-G is associated with better heart graft tolerance along with the present demonstration that HLA-G can be secreted by $CD4^+$ allo-specific T cells after *in vitro* allogeneic reactions, we propose that soluble HLA-G may be a factor limiting allo-responses, thus potentially constituting a new agent for controlling allograft rejection.

We thank Drs. Daniel Geraghty and Michael McMaster for generously providing us with HLA-G antibodies, Béatrice Riteau for having established the M8 transfectants, Catherine Menier and Jean-François Bourge for their helpful advice in cytofluorometry, and Noah Hardy for having edited the manuscript. This study was supported by grants from the association Vaincre la Mucoviscidose, the Etablissement Français des Greffes, the Société Française de Transplantation, and the French Commissariat à l'Energie Atomique.

1. Kovats, S., Main, E. K., Librach, C., Stubblebine, M., Fisher, S. J. & De Mars, R. (1990) *Science* **248,** 220–223.
2. McMaster, M. T., Librach, C., Zhou, Y., Lim, K. H., Janatpour, M. J., De Mars, R., Kovats, S., Damsky, C. & Fisher, S. J. (1995) *J. Immunol.* **154,** 3771–3778.
3. Rouas-Freiss, N., Marchal-Bras Goncalves, R., Menier, C., Dausset, J. & Carosella, E. D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11520–11525.
4. Ishitani, A. & Geraghty, D. E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3947–3951.
5. Fujii, T., Ishitani, A. & Geraghty, D. E. (1994) *J. Immunol.* **153,** 5516–5524.
6. Carosella, E. D., Paul, P., Moreau, P. & Rouas-Freiss, N. (2000) *Immunol. Today* **21,** 532–534.
7. Paul, P., Adrian Cabestre, F., Ibrahim, E. C., Lefebvre, S., Khalil-Daher, I., Vazeux, G., Quiles, R. M., Bermond, F., Dausset, J. & Carosella, E. D. (2000) *Hum. Immunol.* **61,** 1138–1149.
8. Hunt, J. S., Jadhav, L., Chu, W., Geraghty, D. E. & Ober, C. (2000) *Am. J. Obstet. Gyneocol.* **183,** 682–688.
9. Crisa, L., McMaster, M. T., Ishii, J. F., Fisher, S. J. & Salmon, D. R. (1997) *J. Exp. Med.* **186,** 289–298.
10. Moreau, P., Adrian-Cabestre F., Menier, C., Guiard, V., Gourand, L., Dausset, J., Carosella, E. D. & Paul, P. (1999) *Int. Immunol.* **11,** 803–811.
11. Amiot, L., Onno, M., Drénou, B., Monvoisin, C. & Fauchet, R. (1998) *Hum. Immunol.* **59,** 524–528.
12. Onno, M., Le Friec, G., Pangault, C., Amiot, L., Guilloux, V., Drenou, B., Caulet-Maugendre, S., Andre, P. & Fauchet, R. (2000) *J. Immunol.* **61,** 1086–1094.
13. Paul, P., Adrian Cabestre, F., Le Gal, F. A., Khalil-Daher, I., Le Danff, C., Schmid, M., Mercier, S., Avril, M. F., Dausset, J., Guillet, J. G. & Carosella, E. D. (1999) *Cancer Res.* **59,** 1954–1960.
14. Paul, P., Rouas-Freiss, N., Khalil-Daher, I., Moreau, P., Riteau, B., Le Gal, F. A., Avril, M. F., Dausset, J., Guillet. J. G. & Carosella, E. D. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4510–4515.
15. Wagner, S. N., Rebmann, V., Willers, C. P., Gross-Wild, H. & Goos, M. (2000) *Lancet* **356,** 220–221.
16. Fournel, S., Aguerre-Girr, M., Huc, X., Lefant, F., Alam, A., Toubert, A., Bensussan, A. & Le Bouteiller, P. (2000) *J. Immunol.* **164,** 6100–6104.
17. Marchal-Bras Goncalves, R., Rouas-Freiss, N., Conna, F., Choppin, J., Dausset, J., Carosella, E. D., Kirszenbaum, M. & Guillet, J. (2001) *Transplant. Proc.* **33,** 2355–2359.
18. Kapasi, K., Albert, S. E., Yie, M., Zavazavan, N. & Libranch, C. L. (2000) *Immunology* **101,** 191–200.
19. Riteau, B., Rouas-Freiss, N., Menier, C., Paul, P., Dausset, J. & Carosella, E. D. (2001) *J. Immunol.* **166,** 5018–5026.
20. Riteau, B., Menier, C., Khalil-Daher, I., Sedlik, C., Dausset, J., Rouas-Freiss, N. & Carosella, E. D. (1999) *J. Reprod. Immunol.* **43,** 203–211.
21. Bainbridge, D., Ellis, S. A. & Sargent, I. L. (2000) *J. Reprod. Immunol.* **48,** 17–26.
22. Kanai, T., Fujii, T., Kozuma, S., Yamashita, T., Miki, A., Kikuchi, A. & Taketani, Y. (2001) *Hum. Reprod.* **7,** 195–200.
23. Kanai, T., Fujii, T., Unno, N., Yamashita, T., Hyodo, H., Miki, A., Hamai, Y., Kozuma, S. & Taketani, Y. (2001) *Am. J. Reprod. Immunol.* **45,** 94–99.
24. Rajagopalan, S. & Long, E. O. (1999) *J. Exp. Med.* **189,** 1093–1100.
25. Ponte, M., Cantoni, R., Biassoni, A., Tradori-Cappai, A., Bentivoglio, G., Vitale, C., Berton, S., Moretta, L. & Mingari, M. C. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 5674–5679.
26. Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M. & Lopez-Botet, M. (1997) *J. Exp. Med.* **186,** 1809–1818.
27. Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L. & Hsu, M. L. (1997) *Immunity* **7,** 273–282.
28. Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R. L., O'Callaghan, C. A., Dunbar, R., Ogg, G. S., Gerundolo, V. & Rolink, A. (1998) *J. Immunol.* **160,** 3096–3100.
29. Lila, N., Carpentier, A., Amrein, C., Khalil-Daher, I., Dausset, J. & Carosella, E. D. (2000) *Lancet* **355,** 2138.
30. Carosella, E. D., Bensussan, A., Dausset, J. & Sasportes, M. (1981) *Medicina* (*B. Aires*) **41,** 65–76.
31. Sasportes, M., Carosella, E. D., Bensussan, A., Mihaesco, C. & Dausset, J. (1981) *J. Immunogenet.* **14,** 117–127.
32. Chu, W., Fant, M. E., Geraghty, D. E. & Hunt, J. S. (1998) *Hum. Immunol.* **59,** 435–442.
33. Rebmann, V., Vanderven, K., Passler, M., Pfeiffer, K., Krebs, D. & Grosse-Wilde, H. (2001) *Tissue Antigens* **57,** 15–21.
34. Yang, Y., Chu, W., Geraghty, D. E. & Hunt, J. S. (1996) *J. Immunol.* **156,** 4224–4231.
35. Lefebvre, S., Adrian Cabestre, F., Moreau, P., Gourand, L., Dausset, J., Berrih-Aknin, S., Carosella E. D. & Paul, P. (2000) *Hum. Immunol.* **11,** 1095–1101.
36. Lefebvre, S., Berrih-Aknin, S., Adrian Cabestre, F., Moreau, P., Poea, S., Gourand, L., Dausset, J., Carosella, E. D. & Paul, P. (2001) *J. Biol. Chem.* **276,** 6133–6139.