A HIGH PROPORTION OF T LYMPHOCYTES THAT INFILTRATE H-2-INCOMPATIBLE HEART ALLOGRAFTS IN VIVO EXPRESS GENES ENCODING CYTOTOXIC CELL-SPECIFIC SERINE PROTEASES, BUT DO NOT EXPRESS THE MEL-14-DEFINED LYMPH NODE HOMING RECEPTOR

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The cellular basis for allograft rejection in vertebrates is not yet understood. After placement of a allograft, a nonrandom subset of blood cells enter these grafts (1–3); among the infiltrating cells, T lymphocytes are believed to be necessary and sufficient for graft rejection (4). Attempts to identify the central T cell effector subset by adoptive transfer of phenotypically defined T cell subsets (i.e., $CD4^+$ or $CD8^+$) have not clarified the issue, perhaps because of an imperfect correlation of surface phenotype with T cell effector functions, such as help, killing, and inflammation (5–7). Thus, one must attempt to analyze the T cell contribution by function rather than phenotype in order to gain a better understanding of allograft rejection.

The knowledge of the molecular effector mechanisms in a graft rejection in vivo is still limited and based mainly on observations made in vitro with cultured cytotoxic cell lines. Cloned CTL and NK cells contain characteristic cytoplasmic granules that contain a pore-forming protein perforin/cytolysin (8–10), as well as proteoglycans (11) and neutral serine esterases (12, 13). Perforin, which shows some structural similarity to the complement component C9 (14, 15), can polymerize in the presence of Ca^{2+} ions to form a ring-like structure with hemolytic properties (9, 10, 16). The granule serine esterases have also been implicated in cell-mediated cytolysis because killing of target cells by CTLs was inhibited with protease inhibitors, or chymotrypsin and trypsin ester substrates (17, 18). Recently, two genes, HF and C11 (also named CTLA-1 and CTLA-3), have been cloned that are selectively expressed in cytotoxic cells (19–21). The HF gene encodes a trypsin-like serine esterase while the C11 gene encodes a serine esterase with apparent specificity for acidic residues (22). The HF protein,

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also called granzyme A (23), is secreted rapidly both from cytolytic $CD4^+$ and $CD8^+$ T cells after stimulation with an appropriate target in vitro. The observed kinetics of serine esterase release and of the delivery of the lethal hit are consistent with the hypothesis that both events occur at the same time (24). Although expression of these serine esterase transcripts correlates well with killer cell function in vitro (19, 20), it was conceivable that they are expressed as the result of nonphysiological stimulation conditions.

The goal of this study was to begin to assess the distribution and the immune functions of CTL serine esterase–expressing cells in vivo in allograft rejection. Therefore, we decided to examine the expression of these two serine esterase genes during an allograft rejection in vivo by in situ hybridization. We also addressed a second issue in this study; normal, nonactivated T and B lymphocytes circulate from blood to lymphoid organs via recognition of lymphoid organ-specific postcapillary high-walled endothelial venules ligands with lymphocyte cell surface homing receptors (25). In mice, the lymph node homing receptor contains an antigenic determinant detected by the mAb MEL-14 (26). Most antigen- and mitogen-activated murine T and B lymphocytes rapidly lose the expression of lymphoid organ homing receptors and the MEL-14 epitope (27). We have proposed elsewhere that loss of lymphoid organ homing receptors might enable activated lymphoid cells to participate in extralymphoidal immune and inflammatory infiltrates without removal by binding to lymphoid organ high-walled endothelial venules, for which they are most avid (28). In that view, lymphocytes participating in allograft rejection infiltrates should be $MEL\text{-}14^-$.

Materials and Methods

Animals. C57BL/Ka and BALB/cJ mice were kept under conventional conditions in our colonies at Stanford University.

Transplantation Technique. Transplantations were performed as previously described in detail (29). In short, the myocardium of newborn (12–36 h) BALB/cJ ($H\text{-}2^d$) donor mice were diced into 0.1–0.2-cm fragments and subsequently transplanted under the kidney capsule of adult (6–8 wk) sex-matched C57 Bl/Ka recipients ($H\text{-}2^b$; experimental animals). As a control, adult BALB/cJ ($H\text{-}2^d$) mice received grafts from the same donor animals under the kidney capsule. On days 2, 4, 6, 8, 10, and 12 after transplantation, three experimental and two control animals were killed and 5-μm frozen sections through the graft were prepared.

Antibodies. Rat mAbs GK1.5 (anti-L3T4), 53.2.1 (anti-Thy 1.2), and 53.6.7 (anti-Lyt-2) were obtained from the American Type Culture Collection, Rockville, MD. Serum-free culture supernatants of these antibodies and of MEL-14 (26) were concentrated by ammonium sulfate precipitation.

Immunohistochemistry. Sections were stained with the mAbs 53.2.1 (anti–Thy-1.2), 53.6.7 (anti–Lyt-2), and GK 1.5 (anti-L3T4) as a first stage and peroxidase-conjugated rabbit anti–rat Igs (DAKO, Denmark), used at a dilution of 1:10 in 10% normal mouse serum or a FITC-conjugated goat antirat antiserum (Caltag Laboratories, South San Francisco, CA) used at 1:10 with 10% normal mouse serum as a second stage reagent. 3,3′-Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as a substrate for the peroxidase. Tissue sections from thymus and spleen were included as a positive control for the staining of the T cell differentiation antigens.

Isolation of Infiltrating Cells from Allografts. The inflammatory area around the initial site of the allograft was excised with a scalpel and teased into small pieces with two forceps. After digestion with collagenase (125 U/ml; Worthington Diagnostic Systems, Inc., Freehold, NJ) for 1 h at 37°C, the remaining pieces of tissue were vigorously pipetted to obtain a cell suspension for subsequent staining and cell sorting.

Preparation of Cells for FACS Analysis. Isolated cells were reacted on ice with fluoresceinated anti-Thy1.2 antibody together with biotinylated rat mAbs specific for the T cell differentiation marker L3T4 or Lyt-2. After a wash in HBSS through a cushion of newborn calf serum, the cells were incubated with Texas Red–coupled avidin (Cappel Laboratories, Cochranville, PA). After a second wash, the cells were resuspended for FACS separation. Debris, erythrocytes, doublets, and dead cells were excluded by forward light scatter and propidium iodide gating.

Multiparameter Cell Sorting. The dual laser FACS used in this experiment was modified from a Becton Dickinson & Co. (Mountain View, CA) machine as described (30). This instrument and supporting computer hardware and software were made available through the FACS-shared users group at Stanford University.

Preparation of Labeled Probe. A 750-bp cDNA fragment of the murine HF^- gene and a 1.1-kb fragment of the $C11^-$ gene were subcloned into the polylinker of the transcription vector pSPT 672 using standard techniques. This vector has a SP6 and a T7 promotor at the 5′ and 3′ end of the multicloning site, respectively. After linearization of the vector with an appropriate restriction enzyme, sense and antisense probes were prepared using SP6-polymerase and $T7^-$ polymerase (both from New England Biolab, Beverly, MA) reactions and (S-35) UTP No. SJ 1303, Amersham Corp., Arlington Heights, IL) at a final concentration of 12 μM. The labeled nucleotide was dried down before adding the other reagents of the reaction mixture. A typical reaction (35 μl) contained 7 μl 5× SP6 buffer (final concentration: 40 mM Tris-HCl, pH 7.9; 6 mM $MgCl_2$; 2 mM spermidine); 3.5 μl 100 mM DTT; 3.5 μl ribonucleotides (CTP, ATP, and GTP; 10 mM each, in 10 mM Hepes, pH 7.4); 3.5 μl BSA, 5 mg/ml; 1 μl Rnasin, 40 U/μl (Promega Biotech., Madison, WI); 1 μl SP6 (or T7) polymerase, 20 U/μl (New England Biolab); 1 μl linearized DNA template, 1 μg/μl; 13.5 μl H_2O. SP6 and T7 reactions were incubated for 90 min at 40°C and 37°C, respectively. DNA template was digested with DNase I (2 U/μg DNA; Worthington) for 15 min at 37°C. The RNA probe was subsequently extracted with phenol/chloroform, separated on a Bio-Gel P-60 spin column, and ethanol precipitated after adding 7.5 μg of yeast tRNA per 10^6 cpm-labeled probe. The probe was subsequently resuspended at 2×10^9 cpm/μl in TE, boiled for 2 min, and stored frozen at −70°C. For the hybridization, this probe was mixed with formamide (final concentration 50%), dextran sulfate (10%), DTT (100 mM), NaCl (300 mM), Tris-HCl, pH 7.5 (20 mM), EDTA (5 mM) Denhardt's solution (1×) at a concentration of 2×10^6 cpm/μl hybridization solution.

In Situ Hybridization. The protocol of Angerer et al. (31) was used with some modifications; 5-μm-thick cryostat sections were placed on poly-L-lysine (Sigma Chemical Co.)–coated glass slides and fixed in 4% paraformaldehyde dissolved in 1× PBS for 20 min, rinsed in PBS, and dehydrated through graded ethanol. Slides were stored at this stage at 4°C before being used for in situ hybridization. In situ hybridizations on different cell populations were done on sorted cells that were spun onto poly-L-lysine–coated glass slides with a Shandon cytocentrifuge. These cytospin preparations were fixed and hybridized as described for cryostat sections. The fixed sections or cytospin preparations were treated with proteinase K (Boehringer Mannheim, Federal Republic of Germany), 1 μg/ml in 100 mM Tris-HCl, pH 8.0, and 50 mM EDTA at 37°C for 30 min. The slides were postfixed again with 4% paraformaldehyde for 20 min. Free amino groups on tissue sections were acetylated by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. For the hybridization step, 10 μl of the hybridization solution (described above) containing 10^6 cpm S-35 UTP-labeled RNA probe were placed on each section, covered with a siliconized coverslip (18 × 18 mm), and sealed with rubber cement. The sections were hybridized at 46°C for 16–18 h. Thereafter, the slides were washed in a solution containing 50% formamide, 2× SSC, 20 mM Tris, pH 7.5, and 5 mM EDTA in four changes for a total of 2 h at 56°C. After the first wash a digestion step with RNase A (20 μg/ml) and RNase T1 (1 U/ml) (both obtained from Sigma Chemical Co.) for 30 min at 37°C was included. The slides were dipped into NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY), 1:2 diluted with 600 mM ammonium acetate, and exposed at 4°C for 8 d. The slides were developed with Kodak developer D-19 for 2.5 min and fixed

TABLE I

Percentage of Different T Cell Subpopulations in the Inflammatory Infiltrate*

Days after transplantation	Allogeneic graft			Syngeneic graft		
	L3T4	LYT-2	MEL-14	L3T4	LYT-2	MEL-14
2	43 ± 21	57 ± 21	11 ± 9	63 ± 18	37 ± 18	ND
4	30 ± 10	70 ± 10	5 ± 1	53 ± 4	47 ± 4	ND
6	23 ± 10	77 ± 10	3 ± 2	53 ± 4	47 ± 4	ND
8	27 ± 10	73 ± 10	1 ± 1	60 ± 7	40 ± 7	ND
10	37 ± 8	63 ± 8	2 ± 3	60 ± 0	40 ± 0	ND
12	27 ± 3	73 ± 3	2 ± 3	58 ± 4	42 ± 4	ND

* Of the Thy-1.2$^+$ infiltrating cells.

with Kodak fixer for 5 min. Counterstaining was done with 4% Giemsa stain (Fisher Scientific Co., Orangeburg, NY) for 10–15 min. From each animal, two sections were each hybridized with a labeled HF and C11 antisense probe (complementary sequence to the cytoplasmic HF and C11 mRNA) and one section was each hybridized with a labeled HF and C11 sense probe.

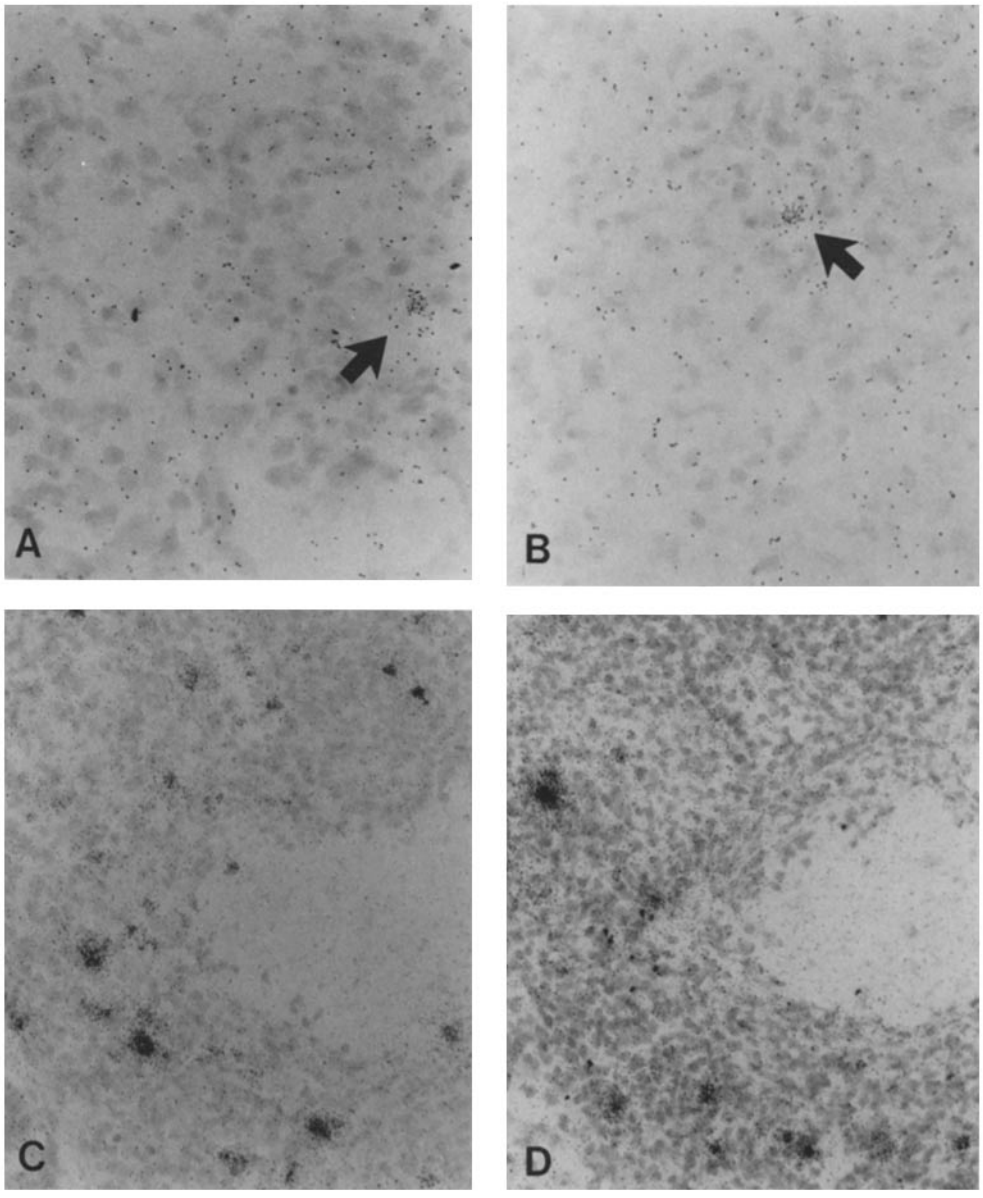
Evaluation of Slides. Cells were considered as positive for gene expression when they had at least twice as many grains as the cells with the highest background on control slides that were hybridized with a labeled sense probe (identical sequence to the cellular target mRNA).

Morphometry. The areas of the inflammatory infiltration at the site of the allogeneic and syngeneic graft were measured using a Hewlett-Packard 9874A digitizer.

Results

The Cellular Infiltrate in Rejecting Allografts Contains T Cells of Both Major Phenotypic Classes (CD4$^+$ and CD8$^+$), and Are MEL-14$^-$. After transplantation, a massive infiltration occurred at the site of the graft in animals with an allogeneic, and to a lesser extent also in animals with a syngeneic, graft. The inflammatory infiltration was maximal around day 10–12 in allogeneic experimental and day 6 in syngeneic control animals; the allografts were completely rejected between days 8 and 12 after transplantation. The phenotypic analysis of the T cell subsets involved in the inflammatory reaction after transplantation showed an initial drop of the CD4$^+$/CD8$^+$ ratio (L3T4/Lyt-2 ratio) in the experimental animals, whereas no significant change was observed in the control animals (Table I). The CD8$^+$ cells were typically clustered around the graft in the experimental animals whereas CD4$^+$ cells were evenly scattered throughout the entire infiltrated area at timepoints later than ~4 d after transplantation. MEL-14$^+$ T cells were most frequently found early after transplantation but represented only a minor T cell subpopulation throughout the rest of the observation period (Table I).

The Cellular Infiltrate in Rejecting Allografts Contains High Proportions of Cells Expressing HF and/or C11 Transcripts. The results of in situ hybridizations with HF- and C11-specific probes are shown in Table II and Fig. 1. The first cells with detectable levels of HF mRNA or C11 mRNA were found on day 2 after transplantation both in animals with an allogeneic and those with a syngeneic graft. These positive cells, however, were extremely rare at this timepoint and were normally not found on every section of the same animal. On day 4 after transplantation, the experimental animals showed a 5–10-fold higher frequency



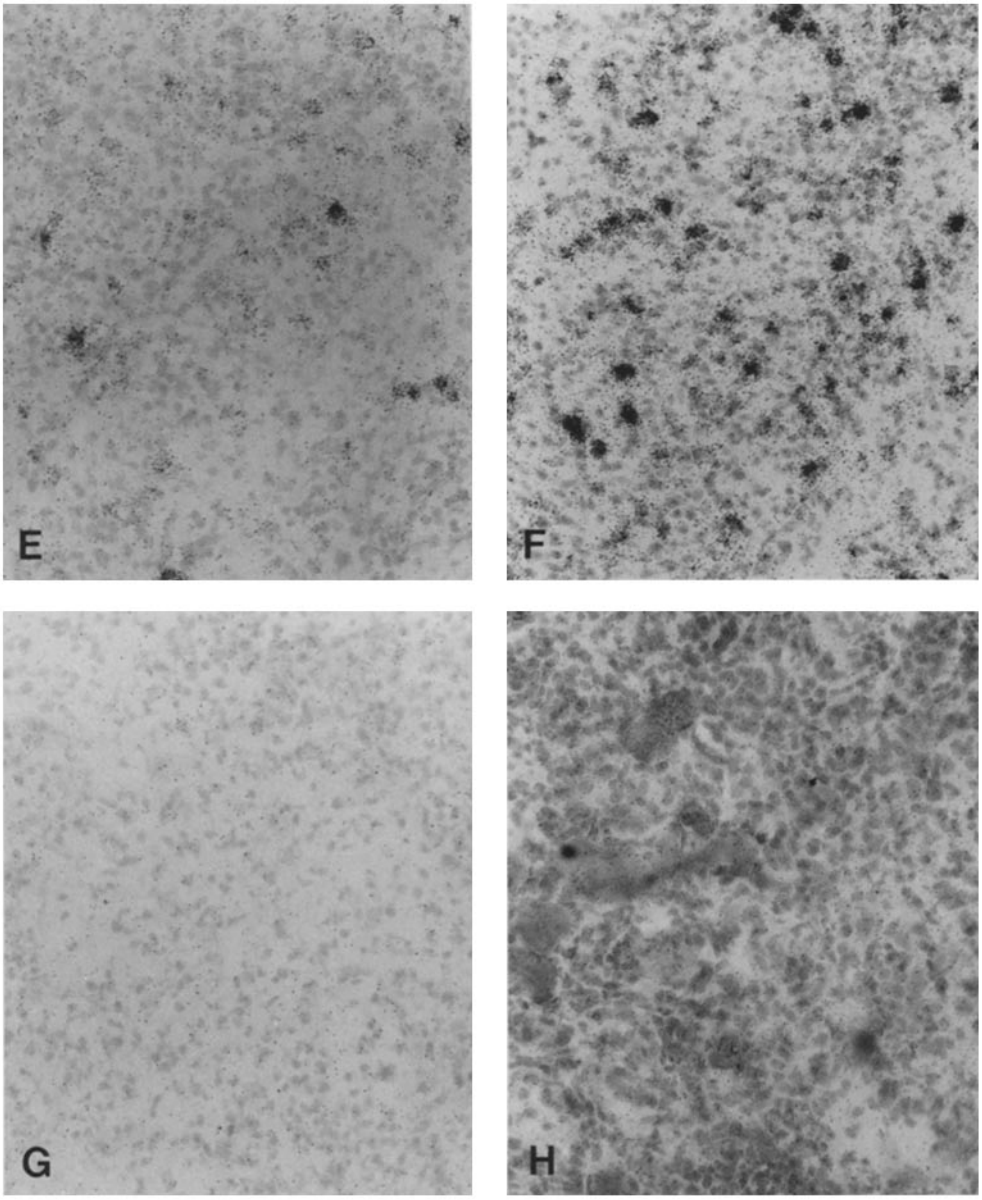


FIGURE 1. Expression of the serine esterase genes HF and C11 during an allograft rejection. Cryostat sections of the graft were prepared 2 (*A* and *B*), 6 (*C* and *D*), and 10 d (*E–G*) after allotransplantation and hybridized with a ^{35}S-labeled antisense riboprobe of the HF (*A*, *C*, and *E*), the C11 gene (*B*, *D*, and *F*), or a ^{35}S-labeled sense probe of the C11 gene (*G*). A cryostat section from a control animal that received a syngeneic graft 6 d before was hybridized with a ^{35}S-labeled antisense riboprobe of the C11 gene (*H*). Arrows in *A* and *B* indicate cells with low levels of serine esterase gene transcripts. (*A* and *B*) ×400; (*C–G*) ×200.

TABLE II
Frequency of Infiltrating Cells with Detectable Levels of HF and C11 mRNA

Days after transplantation	Allogeneic graft		Syngeneic graft	
	HF	C11	HF	C11
2	2 ± 0.8	3 ± 4	7 ± 3	4 ± 1
4	61 ± 94	44 ± 69	3 ± 1	3 ± 4
6	187 ± 58	205 ± 84	19 ± 18	7 ± 4
8	214 ± 64	313 ± 56	22 ± 6	21 ± 12
10	177 ± 46	323 ± 112	24 ± 6	15 ± 1
12	270 ± 171	350 ± 189	6 ± 1	3 ± 1

Frequency of infiltrating cells with detectable levels of HF and C11 mRNA. Cryostat sections of the graft were hybridized with a radiolabeled RNA antisense probe of the HF and the C11 gene, respectively. The figures represent the number of positive cells per unit area (1 mm^2) of infiltration area. Three animals with an allograft and two animals with a syngeneic graft were examined and two sections of each animal and each probe were used for evaluation.

of HF^+ or $C11^+$ cells than the control group with a syngeneic graft. The frequency of inflammatory cells expressing the two serine esterase genes increased dramatically between day 4 and 12 after allotransplantation and was at least eight times higher than in the control animals during this period.

In one of the control animals, the syngeneic graft became necrotic and no viable syngeneic graft cells could be detected 8 d after transplantation. This animal, which was not included in Table I, had 5–10 times more HF or C11 $mRNA^+$ cells than other control animals at the same timepoint. However, compared to the experimental animals 8 d after transplantation, the frequency of positive cells was still ~50% lower. In the first 4 d after the mice received the allograft, about equal numbers of HF^+ and $C11^+$ cells were found among the infiltrating cells. Afterwards, cells with detectable levels of C11 mRNA became slightly more frequent than those with HF mRNA.

The amount of serine esterase–specific mRNA per cell, as measured as the number of silver grains over a single cell, increased steadily during the entire observation period in the experimental animals, indicating that the two genes were expressed over long periods, perhaps after local induction by alloantigen and/or mediators (Table III). The number of grains per cell was generally higher after hybridization with the C11 probe rather than with the HF^- probe. In control animals, the expression level of both serine esterases increased only slightly after more than 4 d after transplantation.

The $HF^+/C11^+$ Cells in Rejecting Allografts Are Mainly in the $CD8^+$ Subset. To estimate reliably the phenotype of HF and C11 transcript-positive cells, small pieces of the allograft were digested with collagenase, and the resulting suspension of the isolated infiltrating T cells were sorted on a FACS for subsequent in situ hybridization. The results of such a sort are shown in Table IV. The infiltrating cells of the allograft and the splenocytes of six animals that received a heart muscle graft 6 d before were pooled and sorted according to their phenotype. The frequency of serine esterase–positive cells in the $CD8^+$ subset was generally 10–20 times higher in the infiltrate of the allograft than in the

TABLE III

Maximal Expression Level of HF and C11 Gene in an Allograft and a Syngeneic Graft*

Days after transplantation	Allogeneic graft		Syngeneic graft	
	HF	C11	HF	C11
2	23 ± 11	22 ± 10	32 ± 25	30 ± 7
4	42 ± 34	54 ± 44	65 ± 50	38 ± 4
6	61 ± 18	87 ± 28	68 ± 28	125 ± 35
8	190 ± 90	420 ± 275	74 ± 37	53 ± 24
10	220 ± 35	610 ± 160	100 ± 7	83 ± 25
12	325 ± 125	770 ± 210	98 ± 8	110 ± 42

* As number of silver grains over a cell after hybridization with a radiolabeled HF and C11 probe, respectively. The four cells with the highest number of silver grains on each tissue section were counted. Two sections per animal and each probe were considered for evaluation.

TABLE IV

Frequency of Serine Esterase–positive Cells among Different Subsets from Spleen and Allograft 6 d after Transplantation

Source	Cell subset	HF mRNA$^+$	C11 mRNA$^+$
Spleen	Thy-1.2$^+$, CD4$^+$	5/1,690 0.3%	2/1,146 0.17%
Spleen	Thy-1.2$^+$, CD8$^+$	2/420 0.48%	5/981 0.51%
Infiltrate of allograft	Thy-1.2$^+$, CD4$^+$	2/415 0.48%	2/149 1.3%
Infiltrate of allograft	Thy-1.2$^+$, CD8$^+$	32/282 11.3%	82/579 14.2%
Infiltrate of allograft	Thy-1.2$^-$	0/201 0%	0/438 0%

Cells infiltrating the graft and spleen cells from animals that received an allograft 6 d before were sorted on the FACS. Cytospin preparations of these cell populations were subsequently hybridized with ^{35}S-labeled probes of the HF and the C11 gene as described in Materials and Methods.

spleen of the same animals. The recovery of CD4$^+$ cells from the infiltrate was always very low and the frequency of serine esterase–positive cells in this subpopulation was at least 10-fold lower than in the CD8$^+$ subset of infiltrating cells; of 34 HF mRNA$^+$ cells analyzed, 32 were CD8$^+$ (94%) and 2 were CD4$^+$ (6%), while of 84 C11 mRNA$^+$ cells analyzed, 82 were CD8$^+$ (98%) and 2 were CD4$^+$ (2%). On cytospin preparations from sorted cells, serine esterase–transcript positive cells were mainly found among the blast-like CD8$^+$ cells. In double stainings of cell suspensions and tissue sections, no evidence for a significant contribution of CD4$^-$,CD8$^-$ or CD4$^+$,CD8$^+$ T cells among the allograft infiltrating cells and the HF or C11 transcript positive cells were found. Interestingly, almost all of the sorted Thy$^+$CD4$^-$,CD8$^-$ cells isolated from allografts turned

out to be nonlymphoid cells that passively acquired Thy-1 antigen that was shed from the surface of the infiltrating T cells (data not shown). Because the efficiency of obtaining infiltrating cells is not exactly known, we hoped to get some information about the phenotype of the cells containing transcripts for the two serine esterases on serial sections that were alternately stained with anti-L3T4 (or anti–Lyt-2) and hybridized with a probe for HF (or C11). This turned out to be difficult, mainly because of the impaired morphology of the sections after in situ hybridization. When we combined immunohistochemistry (with alkaline phosphatase as a detection system) with subsequent in situ hybridization, we observed a significant loss of the signal and an increased background to the in situ hybridization, thus considerably decreasing the sensitivity for detection of serine esterase transcripts.

Discussion

Studies on the interaction of cultured cytotoxic cell lines with their target cells have contributed considerably to our knowledge of the molecular mechanism of cell-mediated cytotoxicity. It is becoming clear, however, that the mechanisms observed during a cell-mediated cytolysis in vitro may or may not exactly reflect the situation in vivo. Berke and Rosen (32) could not detect perforin in in vivo–primed peritoneal exudate cytotoxic T lymphocytes electron microscopically or on immunoblots with a polyclonal antiserum against perforin. While this could be due to quantitative problems, sampling problems, or a characteristic of this class of killer cells, these (and other) findings required an objective, quantitative search for cells in vivo that express genes whose products are found in cytolytic granules. Therefore, we decided to examine the expression of the HF and the C11 genes in vivo during an allograft rejection by doing in situ hybridizations on cryostat sections. 2 d after transplantation, very few scattered cells with detectable levels of transcripts for the HF or the C11 gene were found both in allografts and syngeneic graft. This is consistent with the finding in vitro that cytotoxic cells can be stimulated to express HF or C11 mRNA 2 d later. Alternatively, these positive cells in the infiltrate might have been induced to express the serine esterase genes by stimulation with allograft-unrelated antigens in the periphery and were subsequently attracted as activated cells to the inflammatory site.

The marked increase in the frequency of cells with detectable levels of transcripts for the HF or C11 gene between day 2 and 6 after transplantation is consistent with the finding of other groups that the allograft-specific cytotoxicity of the infiltrating cells increases dramatically between days 4 and 6 after transplantation (33). The kinetics of the appearance of the HF^+ and C11 $mRNA^+$ cells also corresponds to the observed histological rejection of the allografts that was completed between days 8 and 12 after transplantation. The question remains as to whether the increased number and frequency of HF and C11 $mRNA^+$ cells represents a local expansion of these cells and/or a selective immigration of specifically activated cells from the periphery.

The level of HF and C11 transcripts, as measured by the number of silver grains per cell increased during the graft rejection process, thus indicating an induction of the gene by repeated stimulation of these cells and/or a decreased

degradation rate for these transcription products. Generally, sections hybridized with a labeled C11 probe showed more silver grains per single positive cell than sections that were hybridized with a labeled HF probe of the same activity. This may in fact represent real differences in the transcription or degradation rate of the two genes after stimulation although other explanations are possible. (*a*) The signal obtained in an in situ hybridization is not only dependent on the frequency of the target mRNA but also from other parameters; e.g., the length of the labeled probe fragments or the thickness of the tissue section. (*b*) The C11 probe may also detect mRNA from C11-related serine esterases that do not cross-hybridize with the HF probe. Indications for the presence of a C11-related esterase gene in cytotoxic cells were found during the cloning of C11 where a fragment of another related serine esterase gene, named B10, was cloned (20) and the analysis of the contents of the cytolytic granules of cultured CTLs revealed eight serine esterases named granzyme A-H, three of them (granzyme B, G, and H) are closely related to the C11 gene (23).

A clear assignment of cell surface phenotype to the cells that express the serine esterase genes was not possible on serial sections. With the combination of immunohistochemical detection of surface antigens and in situ hybridization, we were able to detect only a small fraction of cells positive for HF or C11 mRNA compared to sections that were hybridized without prior immunohistochemistry. Therefore, we sorted the isolated infiltrating cells on a FACS according to their CD4 and CD8 phenotype for subsequent in situ hybridization with an HF or C11 probe.

The results obtained with sorted cells show that the $CD8^+$ cells in the infiltrate are highly enriched for cells with transcripts for both serine esterases compared to $CD8^+$ T cells in the spleen. Based on the estimated cellularity of ~4,000 cells/mm^2 tissue section, we calculated the percentage of serine esterase transcript–positive cells in the infiltrate as 4–8% between 6 and 12 d after transplantation. These figures may represent an underestimate since the sensitivity of the in situ hybridization is generally lower on tissue sections than on cell suspensions but are still considerably higher than the cell fraction (0.2%) that was found to have immunological specificity for the allogeneic cells in a sponge matrix model (1). In Northern blot analysis, transcripts of the C11 gene, but not for the HF gene were also found in cultured mast cells (34). We could find no histological evidence for a significant infiltration of the allograft with mast cells (data not shown).

The question of whether these 4–8% of the infiltrating cells with serine esterase transcripts can reject an allograft by itself or whether other cell types and other effector mechanism are required for the completion of a rejection, cannot be answered conclusively with this experiment. It may be of interest, however, that low numbers of $CD4^+$ T cells specific for myelin basic protein can induce experimental allergic encephalomyelitis in irradiated rats (35), implying that a small number of cells is capable of inducing tissue damage; the function of the large majority of infiltrating cells seen in an unmodified host remains to be determined for both experimental allergic encephalomyelitis and allograft rejection.

Resting small lymphocytes that contain precursors of CTL are mainly circulat-

ing, MEL-14$^+$,CD8$^+$, serine esterase–negative cells. The phenotype of the infiltrated cells is mainly CD8$^+$,Mel 14$^-$,HF$^+$/C11$^+$ large lymphocytes. It remains to be determined whether the phenotypic transformation these cells must undergo occurs in draining lymph nodes or in the graft itself. If it occurs in the lymph node then the nonrandom accumulation of these cells in the allograft implies a new type of homing specificity, not dependent on the lymph node homing receptor defined by MEL-14.

Summary

The role of cytotoxic cells in in vivo immune functions such as allograft rejection is unknown. To begin to assess the function of cytolytic cells in vivo we have begun with cytolytic cell–specific functional molecules: we have isolated and characterized cytolytic cell–specific cDNA clones from cytolytic T cell clones, both encoding distinct serine esterases. The HF gene encodes a trypsin-like enzyme while the C11 gene encodes an enzyme with likely specificity for acidic residues. Here we demonstrate, using in situ hybridization with RNA probe, that both genes are expressed selectively in a subset of T lymphocytes that have infiltrated cardiac allografts. The phenotype of these cells is consistent with the most frequent phenotype of active CTL raised in vitro: they are predominantly CD4$^-$,CD8$^+$,MEL-14$^-$ T cell blasts. Thus the expression of these genes, each of which encodes serine esterase found in killer cell granules in vitro, is a valid marker for these cells in vivo as well. The kinetics of their accumulation is consistent with, but not proof of, a putative role in allograft rejection. It is likely that HF and C11 gene expression will be of diagnostic value.

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