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*J Immunol* 1998; 161:2151-2157;
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Gallium Arsenide Modulates Proteolytic Cathepsin Activities and Antigen Processing by Macrophages

Timothy A. Lewis, Constance B. Hartmann, and Kathleen L. McCoy

Gallium arsenide (GaAs) is a semiconductor utilized in the electronics industry. Chemical exposure of animals causes a local inflammatory reaction, but systemic immunosuppression. Mice were administered i.p. 200 mg/kg GaAs crystals or latex beads, or vehicle. Five days after exposure, splenic macrophages were defective, whereas thioglycolate-elicited peritoneal macrophages (PEC) were more efficient in processing the Ag, pigeon cytochrome c, than vehicle control macrophages. Various aspects of the MHC class II Ag-processing pathway were examined. Both macrophage populations normally presented a peptide fragment to the CD4+ T cells. Surface MHC class II expression on the PEC was up-regulated, but splenic cells had normal MHC class II expression. PEC had elevated levels of glutathione and cysteine, major physiologic reducing thiols. However, the cysteine content of splenic macrophages was diminished. Proteolytic activities of aspartyl cathepsin D, and thiol cathepsins B and L were decreased significantly in splenic macrophages. On the other hand, thiol cathepsin activities were increased selectively in PEC. Latex bead-exposed PEC were not more potent APC, and their thiol cathepsin activities were unchanged, indicating that phagocytosis and nonspecific irritation were not responsible. The phenotype of PEC directly exposed to GaAs mirrored cytokine-activated macrophages, in contrast to splenic macrophages from a distant site. Therefore, GaAs exposure differentially modulated cathepsin activities in splenic macrophages and PEC, which correlated with their Ag-processing efficiency. Perhaps such distinct alterations may contribute to the local inflammation and systemic immunotoxicity caused by chemical exposure. The Journal of Immunology, 1998, 161: 2151–2157.
Conversely, peritoneal macrophages from the exposure site are more potent APC than either vehicle control- or latex bead-exposed macrophages (45). In both cases, chemically exposed cells present antigenic peptides, which do not require processing, to CD4+ T cells normally (42, 45). Nothing is known about the mechanism by which GaAs impacts Ag processing. In the current study, various aspects of the Ag-processing pathway were examined. The Ag-processing defect exhibited by splenic macrophages was associated with diminished aspartyl and thiol cathepsin activities. In contrast, the potent peritoneal macrophages displayed increased thiol cathepsin activities. We propose that GaAs influences Ag processing, in part by altering proteolytic activities in APC, which may be determined by the chemical form(s) to which the cells are exposed.

Materials and Methods

Mice

Female C3D2F1/J mice (The Jackson Laboratory, Bar Harbor, ME) were used from 9 to 18 wk of age. Mice were housed under specific pathogen-free conditions.

Monoclonal Abs

The mAb-producing B cell hybrids from American Type Culture Collection (ATCC, Manassas, VA) included anti-B220 (clone RA3-3A1), anti-Thy-1.2 (clone J3-10), and anti-rat E-light chain (clone MAR 18.5). Bioconjugated anti-Mac-1 (clone M170), FITC-conjugated anti-I-A\(^d\) (clone AMS 32.1), and FITC-conjugated anti-I-E\(^b\) (clone 17-3-3S) were purchased from Pharmingen (San Diego, CA). FITC-conjugated anti-I-A\(^b\) (clone MRC OX-3) was purchased from SeroTek USA (Washington, DC). Protein A affinity-purified MAR 18.5 was coupled with FITC isomer I (Molecular Probes, Eugene, OR).

Cell lines and cell culture

Pigeon cytochrome c-specific, I-E\(^b\)-restricted T cell hybridoma 2B4.11 was provided by Dr. Ronald Schwartz (National Institutes of Health, Bethesda, MD). The IL-2-dependent cell line CTLL-2 was obtained from ATCC. Culture supernatants from the ribbon T cell line MLA-144 were a source of IL-2 (ATCC). Medium for cell culture was previously described (26).

Chemical exposure

Mice were exposed to a single i.p. injection of GaAs at 200 mg/kg body weight (Research Triangle Institute, Research Triangle Park, NC) or a suspension of particles with a mean diameter of 1.5 \(\mu\)m in saline containing 0.05% Tween-80 (Sigma, St. Louis, MO). Our previous toxicologic studies determined that this GaAs dose modulates immune functions without generalized toxicity (42). In some experiments, mice were administered i.p. 200 mg/kg body weight latex beads (Sigma) with a mean diameter of 1 \(\mu\)m in saline containing 0.05% Tween-80. Control mice received the vehicle consisting of 0.05% Tween-80 in saline. All suspensions were prepared immediately before use. Peritoneal macrophages were induced by i.p. injection of 2 ml of 10% Brewer’s thioglycolate broth (Difco, Detroit, MI), washed with RPMI 1640. Similarly, peritoneal cells were incubated with anti-B220 mAb, followed by C. Cellular composition was assessed by immunofluorescence staining and flow cytometry. No significant differences in the frequency of various cell lineages were detected among the exposure groups. The remaining splenic cells were approximately 62% Mac-1+ cells, <6% B cells, and <2% T cells. The surviving peritoneal cells were nearly 80% Mac-1+ cells, <5% B cells, and <3% T cells. For both splenic and peritoneal cells, <3% of Mac-1+ cells expressed MHC class II molecules.

Preparation of macrophages

Splenocytes at 1 \(\times\) 10^7/ml were incubated with saturating amounts of anti-B220 and anti-Thy-1.2 mAb for 15 min at 4°C. Cells were incubated with rabbit C (Cedarlane, Ontario, CA) at a 1:5 dilution at 37°C for 45 min and washed with RPMI 1640. Similarly, peritoneal cells were incubated with anti-B220 mAb, followed by C. Cellular composition was assessed by immunofluorescence staining and flow cytometry. No significant differences in the frequency of various cell lineages were detected among the exposure groups. The remaining splenic cells were approximately 62% Mac-1+ cells, <6% B cells, and <2% T cells. The surviving peritoneal cells were nearly 80% Mac-1+ cells, <5% B cells, and <3% T cells. For both splenic and peritoneal cells, <3% of Mac-1+ cells expressed MHC class II molecules.

T cell stimulation assay

T cell hybridoma 2B4.11 at 3 \(\times\) 10^5/ml was added to 1 \(\times\) 10^6 macrophage-enriched splenocytes or 2.5 \(\times\) 10^5 PEC in complete medium with or without various concentrations of Ag. Intact pigeon cytochrome c (Sigma), or CNBr cleavage fragment 66-104 of cytochrome, which was prepared as described (46), was the Ag. Cells were cultured in 96-well flat-bottom microtiter plates in triplicate at 37°C for 24 h. Macrophage-enriched splenocytes and PEC were irradiated with 3000 rad using a Cs source before culture to eliminate the Ag-presenting function of other cell populations besides macrophages and to prevent cell proliferation due to cytokines secreted by activated T cells (47). Cell-free culture supernatants were assayed for IL-2 by incubating CTLL-2 cells with 25% culture supernatants for 37°C at 18 h. The wells were pulsed with 1 \(\mu\)Ci[^3H]Tdr (Amersham, Arlington Heights, IL) and harvested after another 6 h by a PHD cell harvester (Cambridge Technologies, Watertown, MA). Radiochemical incorporation was quantitated by liquid scintillation counting. A standard preparation of IL-2 was included as a positive control in the assay.

Immunofluorescence staining and flow-cytometric analysis

Spleen macrophages and PEC were incubated with 25 \(\mu\)g normal mouse IgG (Sigma) to block FcR. To detect Mac-1 expression, cells were incubated with biotinylated M170, followed by phycoerythrin-conjugated streptavidin (Life Technologies, Gaithersburg, MD). To detect I-A\(^d\) and I-E\(^b\) class II molecules, cells were incubated with FITC-conjugated AMS 32.1 or FITC-conjugated control 17-3-3S, respectively. Background control cells incubated with irrelevant FITC-conjugated MRC OX-3, and phycoerythrin-conjugated streptavidin. The fluorescence intensity of viable cells was measured with logarithmic amplification using a Becton Dickinson FACScan (San Jose, CA) equipped with a 15-mW 488-nm argon laser and appropriate excitation filters. Data on 20,000 splenocytes and 10,000 PEC were collected, and forward-angle side scatter gates were set to exclude cell clumps and dead cells.

Intracellular thiol assays

Cell lysates from macrophage-enriched cells at 2.5 \(\times\) 10^5/ml were prepared in 2.5% sulfoacetic acid. The quantity of precipitated protein was measured by a bicinchoninic acid assay (Pierce, Rockford, IL). Intracellular cysteine was measured as described (48). Briefly, supernatants were mixed with nihydrin (Sigma) and heated to 100°C for 10 min. Absorbance at 560 nm was measured with a background control lacking a cell sample. Concentration of intracellular cysteine was calculated from a standard curve of cysteine hydrochloride. Glutathione in the supernatants was assayed as described (49). The reaction mixture contained 5.5-dithiobis(2-nitrobenzoic acid) (Pierce), 10 \(\mu\)g yeast glutathione reductase (Sigma), and 0.2 \(\mu\)M nicotinamide-adenine dinucleotide phosphate (NADPH). Absorbance at 412 nm was measured throughout the reaction period, and rates of reaction were calculated. Background control lacked cell samples. Concentration of intracellular glutathione was calculated from a standard curve of glutathione. Data are expressed as nmol/10^7 cells. In one set of experiments, various concentrations of gallic nitrate, sodium arsenite, or sodium arsinite (Sigma) were added to one concentration of cysteine or glutathione.

Protease assays

Cell lysates were prepared in 0.75% Triton X-100 lysis buffer at 1 \(\times\) 10^8 cells/ml. Protein concentration in the cell lysate was determined by bicinchoninic acid assay. Linearity of protease activities in the lysates was established for both protein concentration and reaction time. Asparty1 cathepsin D activity was measured as described (50). Briefly, samples in duplicate were incubated with 8% hemoglobin in 1 M sodium acetate buffer at pH 4.5 in the absence and presence of 10 \(\mu\)g/ml peptatin A (Sigma) for 2 h at 37°C. Reactions were stopped by 5% TCA, and ninhydrin was added after centrifugation of the samples. After heating the samples to 100°C for 10 min, absorbance at 550 nm was measured with a background control that lacked cell samples. Absorbance in samples containing peptatin A was subtracted from that in samples lacking peptatin A. One unit of activity is a net absorbance of 1. Results are expressed as U/10^7 cells.

The peritoneal macrophages were activated by incubation at 37°C for 10 min before assay (51). The activation buffer for cathepsin B was 87.7 mM KH₂PO₄/12.3 mM Na₂HPO₄ containing 4 mM EDTA and 2.6 mM DTT (Sigma) at pH 6. Cathepsin B activity was measured by cleavage of 25 \(\mu\)M Z-Arg-Arg-aminomethyl coumarin (Bachem, King of Prussia, PA), a specific cathepsin B substrate. The activation buffer for cathepsin L was 340 mM sodium acetate/60 mM acetic acid containing 4 mM EDTA and 1.3 mM DTT at pH 5.5. Cathepsin L activity was assayed by cleavage of 33 \(\mu\)M Z-Phe-Arg-aminomethyl coumarin (Bachem) in the absence and presence
of Z-Phe-Phe-CN₂, (Bachem), a specific cathepsin L inhibitor (51). After 3 h, reactions were stopped by 1 mM iodoacetamide (Sigma). Background controls were reactions lacking cell samples. Fluorescence was measured by a Shimadzu spectrofluorophotometer RF5000 (Columbia, MD) with an excitation wavelength at 370 nm and emission wavelength at 460 nm. The spectrofluorophotometer was calibrated with 7-amino-4-methyl coumarin (Bachem), the reaction product. One unit of activity is a net fluorescence unit of 1. Data are expressed as U/10⁵ cells.

Statistical analyses

Ag dose-response curves were analyzed by factorial ANOVA. Experimental results were tested for homogeneity by Bartlett’s test. Parametric analysis of variance was performed by two-tailed Student’s t test for unmatched pairs. Nonparametric analysis of variance was performed by two-tailed Mann-Whitney test. Experimental treatment groups were compared with the vehicle control, and p values ≤ 0.05 were considered significant.

Results

GaAs exposure alters Ag processing by macrophages

The influence of in vivo GaAs exposure on the ability of macrophages to function as APC was investigated. Macrophages from two different anatomic locations were enriched by mAb and C cytolyis. The frequency of macrophages within the surviving cell populations was not significantly different among the various exposure groups. Ag processing was assessed by stimulation of the cytochrome-specific CD4⁺ Th cell hybridoma to secrete IL-2. GaAs-exposed splenic macrophages were less efficient in processing cytochrome than vehicle control macrophages (Fig. 1A). The Ag dose-response curve was shifted to the right by approximately fivefold, and the maximum was reduced by nearly one-third.

On the other hand, GaAs had the opposite effect on PEC from the exposure site (Fig. 1B). At all cytochrome concentrations examined, GaAs-exposed PEC were more potent APC than vehicle control cells. To examine the contribution of phagocytosis and nonspecific irritation to the enhanced PEC function, mice were administered latex beads with a similar diameter as GaAs before thioglycolate induction. Latex bead-exposed PEC were comparable with vehicle cells in eliciting a T cell response (Fig. 1C), unlike chemically exposed PEC.

The ability of GaAs-exposed macrophages to present a peptide fragment of cytochrome, which does not require processing, was examined (Fig. 2). Neither the splenic nor peritoneal macrophages exposed to GaAs exhibited a significant difference in presenting the peptide 66–104 to the T cells compared with the vehicle control cells. These data suggest that GaAs mediates differential effects on cytochrome processing, but not presentation, depending on the source of the macrophages.

GaAs increases surface MHC class II molecule expression on PEC

CD4⁺ Th cell responses depend on both Ag concentration and MHC class II expression on APC (2). Expression of MHC class II molecules on macrophages (Mac-1⁺ cells) was measured by two-color immunofluorescence staining and flow cytometry. GaAs did not alter the level of MHC I-A² class II expression on splenic macrophages (Fig. 3A). The average mean fluorescence intensities were 31.5 ± 2.7 for vehicle control Mac-1⁺ cells and 31.7 ± 4.1 for GaAs-exposed cells (NS). The percentage of splenic macrophages expressing I-A² molecules was essentially identical between the groups (42 ± 4% and 42 ± 9% for vehicle- and GaAs-exposed cells, respectively). Similar results were observed for MHC I-E² class II molecules (data not shown).

On the other hand, surface MHC I-A² expression was up-regulated on GaAs-exposed PEC (Fig. 3B). The fluorescence intensity profile of chemically exposed PEC was shifted to higher values. The average mean fluorescence intensity was 99 ± 5 for GaAs-exposed Mac-1⁺ cells compared with 54.4 ± 3.9 for vehicle control cells (p < 0.001). A slight, yet significant, increase in the percentage of positive PEC occurred (46 ± 4% for vehicle control cells vs 55 ± 3% for GaAs-exposed cells; p < 0.02). In addition, GaAs-exposed PEC expressed a higher level of MHC I-E² class II molecules than vehicle PEC (data not shown).

GaAs modulates intracellular thiol levels

Intracellular thiol levels in APC correlate with their efficiency to process Ag (31, 32). The quantitation of cysteine and glutathione, major physiologic thiols, within macrophages was measured. GaAs-exposed splenic macrophages had a normal glutathione level on a per cell basis (Table I). However, the cysteine content in

FIGURE 1. GaAs influences Ag processing by macrophages. Pigeon cytochrome c-specific T cell hybridoma 2B4.11 was incubated with the indicated native cytochrome concentrations and with vehicle (○), GaAs (■), or latex bead-exposed (□) macrophage-enriched cells. Macrophages were enriched by mAb and C cytolyis. Culture supernatants were assayed for IL-2. Values are the mean cpm in experimental cultures minus the mean cpm in medium control ± SD for triplicate wells. Each experiment is representative of four. A. Mice were exposed i.p. to vehicle or 200 mg/kg GaAs for 5 days. Splenic macrophages were the APC. Medium controls were 1753 ± 262 cpm for vehicle cells, and 1511 ± 169 cpm for GaAs-exposed cells. Vehicle vs GaAs: p < 0.001. B. Mice were administered vehicle or GaAs and then thioglycolate, 24 h later. PEC were harvested after another 4 days. Medium controls were 1103 ± 257 cpm and 931 ± 214 cpm for vehicle- and GaAs-exposed cells, respectively. Vehicle vs GaAs: p < 0.0001. C. Mice were exposed i.p. to vehicle or 200 mg/kg latex beads, and PEC were obtained as in B. Medium controls were 1327 ± 435 cpm for vehicle cells, and 1122 ± 82 cpm for latex bead-exposed cells. Vehicle vs latex bead: NS.
chemically exposed splenic cells decreased by >fourfold compared with vehicle control cells. In contrast, GaAs-exposed PEC contained significantly elevated amounts of both thiols/10⁷ cells than control PEC (Table I). The differences in thiol content were not associated with changes in protein level. The amount of acid-precipitable protein in the cell lysates was comparable between the exposure groups, although PEC regardless of exposure had a higher protein content than splenic cells (Table I).

Macrophages may have contained GaAs or its soluble components that may have influenced the above measurements. Various concentrations of sodium arsenite, sodium arsenate, and gallium nitrate were added to the glutathione assay with a fixed standard concentration. The highest chemical concentration was 100-fold greater than the standard on a molar basis. None of the chemicals at the tested concentrations altered the results of the standard (Fig. 4A). Arsenite, which binds sulfhydryls (52), was incubated with 80 μM cysteine. The presence of sodium arsenite up to 800 μM did not significantly affect the sensitivity of the cysteine measurement (Fig. 4B). All three chemicals at a molar ratio of 100:1 were added to the protein standard, BSA. Again, the chemicals did not significantly change the measured value for the protein standard (data not shown).

**GaAs changes protease activities**

Cathepsins are endosomal and lysosomal proteases with an acidic pH optimum (50, 51). We measured the enzymatic activity of three major cathepsins: aspartyl cathepsin D, and thiol cathepsins B and L. Analogous to the above results, GaAs did not alter the quantity of detergent-soluble protein recovered from splenic enriched macrophages or PEC (Table II), although PEC had a higher protein content than splenic cells, which was unrelated to chemical exposure. On a per cell basis, GaAs-exposed splenic macrophages contained approximately one-half the cathepsin D activity in whole cell lysates compared with vehicle control cells (Table II). In addition, GaAs-exposed splenic macrophages also had significantly diminished thiol cathepsin activities (Table III). Greater than a twofold reduction in cathepsin B activity was detected in chemically exposed splenic macrophages. Cathepsin L activity in GaAs-exposed splenic macrophages was only 60% of the vehicle level. Thus, the decreased cathepsin activities in GaAs-exposed splenic macrophages may impair their ability to process Ag.

Contrary to these findings, PEC from vehicle- and GaAs-exposed mice displayed nearly equal aspartyl protease activity on a per cell basis (Table II). Unlike splenic macrophages, thiol protease activities were significantly increased in GaAs-exposed PEC (Table III). For cathepsin B, chemically exposed PEC displayed 2.5 higher activity than vehicle PEC, and cathepsin L activity nearly doubled in GaAs-exposed PEC. To determine whether the

| Table 1. GaAs exposure affects thiol content in macrophagesa |
|---------------------------------|-----------------|-----------------|-----------------|
| Source of Macrophages | Glutathione (nmol/10⁷ cells) | Cysteine (nmol/10⁷ cells) | Protein (μg/10⁷ cells) |
| Vehicle spleen | 1.1 ± 0.4 | 13.9 ± 6.7 | 340 ± 188 |
| GaAs spleen | 1.1 ± 0.2 | 3.2 ± 2.7b | 280 ± 60 |
| Vehicle PEC | 1.5 ± 0.9 | 5.9 ± 3.2 | 1052 ± 336 |
| GaAs PEC | 3.5 ± 0.8b | 11.7 ± 7.9b | 1240 ± 580 |

a Cell lysates were prepared in sulfosalicylic acid from macrophage-enriched cells after a 5-day exposure. Glutathione and cysteine in the supernatants and acid-precipitable protein were measured. Values are the mean ± SD of five separate lysates.

b For peritoneal glutathione, vehicle vs GaAs: p < 0.01.

For splenic and peritoneal cysteine, vehicle vs GaAs: p < 0.05.

FIGURE 2. Normal peptide presentation by GaAs-exposed macrophages. T cells were incubated with various concentrations of peptide 66–104 of cytochrome and with vehicle (○) or GaAs (●)-exposed macrophage-enriched cells (see Fig. 1). IL-2 in the culture supernatants was measured, and each experiment is representative of three. A. Splenic macrophages were the APC. Medium controls were 2144 ± 145 cpm and 2477 ± 299 cpm for vehicle- and GaAs-exposed cells, respectively. Vehicle vs GaAs: NS. B. PEC were the APC. Medium controls were 2963 ± 121 cpm for vehicle cells, and 1923 ± 640 cpm for GaAs-exposed cells. Vehicle vs GaAs: NS.

**FIGURE 3.** GaAs exposure up-regulates MHC class II expression on peritoneal, but not splenic, macrophages. Mice were exposed to vehicle or GaAs (see Fig. 1). Cells were stained for MHC I-A<sup>d</sup> class II and Mac-1 molecules, and the fluorescence intensity was collected by two-color flow cytometry. Data are representative green fluorescence profiles of Mac-1<sup>+</sup> cells. A. Splenic Mac-1<sup>+</sup> cells. Vehicle control cells incubated with anti-I-A<sup>d</sup> (—) or control (——) mAb. GaAs-exposed cells incubated with anti-I-A<sup>d</sup> mAb (—). B. Peritoneal Mac-1<sup>+</sup> cells. Vehicle cells stained with anti-I-A<sup>d</sup> (—) or control (——) mAb. GaAs-exposed cells stained with anti-I-A<sup>d</sup> (—) mAb.
augmented thiol cathepsin activities were due to phagocytosis, protease activities in PEC exposed to latex beads were measured. The amount of detergent-soluble protein recovered from latex bead-exposed PEC was 329 ± 73 μg/10⁷ cells, which was not significantly different from protein levels in vehicle- and GaAs-exposed PEC (see Table II). Neither cathepsin B nor L activities were increased significantly in latex bead-exposed PEC compared with the control PEC (Table III), in contrast to GaAs-exposed PEC. Thus, the higher thiol cathepsin activities in GaAs-exposed PEC were not simply due to phagocytosis, but rather a direct result of chemical exposure.

Discussion

In the present study, we investigated various aspects of the Ag-processing pathway within macrophages exposed to GaAs in vivo.

**Table II.** GaAs exposure decreases aspartyl cathepsin D activity in splenic macrophages

<table>
<thead>
<tr>
<th>Source of Macrophages</th>
<th>Protein (μg/10⁷ cells)</th>
<th>Cathepsin D (U/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle spleen</td>
<td>174 ± 58</td>
<td>6.6 ± 2.2</td>
</tr>
<tr>
<td>GaAs spleen</td>
<td>162 ± 53</td>
<td>3.6 ± 1.5</td>
</tr>
<tr>
<td>Vehicle PEC</td>
<td>413 ± 114</td>
<td>35.4 ± 9.7</td>
</tr>
<tr>
<td>GaAs PEC</td>
<td>402 ± 94</td>
<td>33.6 ± 13.6</td>
</tr>
</tbody>
</table>

*Cell lysates were prepared in 0.75% Triton X-100 lysis buffer from day 5 exposed macrophage-enriched cells. Detergent-soluble protein and aspartyl cathepsin D activity in the lysates were measured. Specific activity of hemoglobin cleavage was calculated by subtracting the proteolytic activity in samples containing pepstatin A from that in samples lacking pepstatin A. Values are the mean ± SD of four separate lysates.

**Table III.** GaAs exposure modifies thiol cathepsin activities in macrophages

<table>
<thead>
<tr>
<th>Source of Macrophages</th>
<th>Cathepsin B (U/10⁷ cells)</th>
<th>Cathepsin L (U/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle spleen</td>
<td>70.1 ± 28.3</td>
<td>137.6 ± 26.6</td>
</tr>
<tr>
<td>GaAs spleen</td>
<td>28.6 ± 10.9</td>
<td>82.3 ± 28.9</td>
</tr>
<tr>
<td>Vehicle PEC</td>
<td>444.0 ± 162.9</td>
<td>1658.4 ± 665.2</td>
</tr>
<tr>
<td>Latex bead PEC</td>
<td>590.5 ± 105.0</td>
<td>2180.3 ± 427.5</td>
</tr>
<tr>
<td>GaAs PEC</td>
<td>1060.6 ± 441.1</td>
<td>2938.7 ± 328.9</td>
</tr>
</tbody>
</table>

*Cell lysates were prepared in 0.75% Triton X-100 lysis buffer (see Table II legend). Cathepsin B activity was measured by cleavage of Z-Arg-Arg-aminomethyl coumarin, a specific substrate. Cathepsin L activity was assayed by cleavage of Z-Phe-Arg-aminomethyl coumarin with and without Z-Phe-Phe-CHN₂, a specific cathepsin L inhibitor. Values are the mean ± SD of four separate lysates.

For peritoneal cathepsin B, vehicle vs GaAs; p < 0.03.

For splenic cathepsin L, vehicle vs GaAs; p < 0.05.

For peritoneal cathepsin L, vehicle vs GaAs; p < 0.02.

PEC were obtained from the exposure site, whereas splenic macrophages were located at a distant site. GaAs-exposed splenic macrophages were impaired in processing cytochrome. In contrast, chemically exposed PEC exhibited an enhanced ability to process cytochrome compared with vehicle control cells. However, both macrophage populations normally presented a peptide fragment of cytochrome to the T cells. Although the T cells may have been exposed to GaAs or its components from the macrophages, the distinct results with intact cytochrome and its peptide indicate that the influence of the chemical on T cell activation was at the level of the APC, not the T cells or the IL-2 indicator cells. These findings agree with our previous studies that GaAs exposure causes an Ag-processing defect in splenic macrophages (42), yet augments Ag processing by PEC (45). Another major consequence of chemical exposure was the altered levels of cathepsin activities within the macrophages that correlated with their Ag-processing efficiency. The activity of all three cathepsins examined was diminished in the splenic macrophages, whereas thiol cathepsin activities were selectively up-regulated in PEC. Our results with GaAs-exposed PEC cannot be explained by phagocytosis or non-specific irritation. Latex bead-exposed PEC were not more potent APC than vehicle PEC, and their thiol cathepsin activities were not significantly increased. Thus, the main impact of the chemical on Ag processing may be due to modulation of cathepsin proteolytic activities.

Several studies clearly demonstrate that treatment of APC in culture with protease inhibitors interferes with Ag processing, although the exact outcome depends on the T cell epitope (3, 7–9, 14). Administration of a cathepsin B-specific inhibitor to mice also impairs the development of in vivo primary Ab and T cell responses to hepatitis B Ag (11). None of these studies measured the extent to which cathepsin activity must be lowered to cause an Ag-processing defect. A major loss of proteolytic activity could be detrimental. For example, the absence of cathepsin D in genetically deficient mice is lethal (53). Aspartyl cathepsin D and thiol cathepsins B and L activities were decreased by approximately twofold in the GaAs-exposed splenic macrophages, suggesting that this degree of down-regulation is sufficient to impair Ag processing. Intracellular thiol levels influence Ag processing. Glutathione and cysteine levels in bone marrow macrophages correlate with their ability to process insulin (31). We recently reported that a significant drop in thiol levels in a competent APC renders the cells defective in Ag processing (32). These physiologic reducing agents presumably promote disulfide bond reduction of Ag and maintain the active site cysteine of thiol cathepsins in a reduced state to enhance their proteolytic activity (27–30). Intracellular
cysteine is derived from several sources, including extracellular cysteine and protein degradation (28, 29). The decreased cysteine content of chemically exposed splenic macrophages may be a reflection of diminished proteolysis as a consequence of lower cathepsin activities. The diminished cysteine level may, in turn, contribute to decreased thiol cathepsin activities. Unlike cysteine, the main cellular source of glutathione is biosynthesis, and the plasma membrane does not have a transporter for this thiol (54, 55). The glutathione assay in our study measured total glutathione and did not distinguish between reduced and oxidized glutathione. Perhaps GaAs exposure shifts the balance of the glutathione forms in the splenic macrophages.

Little is known about the regulation of cathepsin expression in macrophages. Diminished proteolysis in the splenic macrophages was not accompanied by a lower total protein content. In addition, their surface expression of MHC class II molecules and total gluthathione content were normal. Thus, a generalized decrease in protein synthesis is unlikely. No GaAs crystals are found within the spleen after i.p. exposure (42). Particulate GaAs dissociates in vivo into its water-soluble components (35–39). Arsenic and gallium enter the circulatory system and are present in the blood and various organs up to 28 days after one exposure (35–39). One study reported that these components accumulate in the spleen over a 14-day time course (39). Simultaneously, the quantity of GaAs diminishes at the exposure site (35–39). Arsenic is viewed as the main immunotoxic component of GaAs (39). Trivalent arsenic is highly reactive and binds monothiols and di thiols (52). Arsenic could directly inhibit thiol cathepsins, similar to the effect of the chemical on other mature cysteine enzymes (52). Aspartyl cathepsin D has a disulfide bridge that stabilizes a loop containing an active site Asp residue (56). If arsenic disrupts this disulfide bridge, the protease would be inactivated. Alternatively, the soluble components may influence the synthesis of cathepsins or their gene transcription. Additional experiments are required to distinguish among these possibilities.

On the other hand, the augmented Ag processing by GaAs-exposed PEC was associated with several phenotypic changes. A higher percentage of GaAs-exposed macrophages were MHC class II+, and the positive cells stained brighter than vehicle control cells. Furthermore, the chemically exposed PEC contained a significantly higher quantity of glutathione and cysteine than vehicle control cells. Finally, the enzymatic activity of the thiol cathepsins, but not the aspartyl cathepsin, was elevated in the GaAs-exposed PEC. All of these characteristics displayed by chemically exposed PEC could improve their efficiency to process Ag. A substantial proportion of PEC phagocytoses GaAs crystals (45), unlike splenic macrophages. However, the enhanced Ag processing by GaAs-exposed macrophages cannot be attributed to phagocytosis alone. Latex bead exposure did not alter the ability of PEC to process Ag, and latex bead-exposed PEC did not have increased thiol cathepsin activities.

The phenotypic features of GaAs-exposed PEC are indicative of an activated state. Up-regulation of MHC class II molecules on the surface of macrophages occurs in response to various stimuli, especially the cytokines IFN-γ and TNF-α (57). Bone marrow macrophages stimulated with granulocyte-macrophage CSF have elevated levels of glutathione and cysteine (31). IFN-γ selectively increases cathepsins B and L, but not cathepsin D, activities in human and murine macrophage cell lines (58). This cytokine also induces the secretion of cathepsin D by human peripheral blood monocytes and alveolar macrophages (59), which may explain the selective nature of the up-regulation thiol cathepsin activities. Furthermore, IFN-γ increases thiol cathepsin H mRNA level in murine PEC; however, other stimuli, including IL-2, IL-4, TNF-α, IL-10, and LPS, have no effect (60). Thus, GaAs-exposed PEC have attributes of cytokine-stimulated macrophages. GaAs may cause a local inflammatory reaction resulting in cytokine secretion at the exposure site, analogous to pulmonary inflammation after intratracheal instillation (35, 36). We are currently investigating the role of cytokines in regulating cathepsin activities in GaAs-exposed macrophages.

The efficiency of Ag processing also depends on the rate of Ag internalization and the intracellular transport pathway (61, 62). Ag that enter APC via receptor-mediated endocytosis are targeted along particular transport pathways, leading to augmented Ag processing (61). Cytochrome and the other Ag used in our previous studies enter APC by fluid-phase pinocytosis, which is a random process and is not receptor mediated. Such molecules, once inside APC, do not follow a distinct transport path and have access to various intracellular compartments, including early and late endosomes, lysosomes, and trans Golgi region (61). The processing defect exhibited by GaAs-exposed splenic macrophages is not global; the cells process some Ag normally (42). If GaAs exposure slowed Ag uptake or altered intracellular transport, one would predict that processing of all Ag would be impaired. On the other hand, GaAs-exposed PEC are more efficient in processing all Ag examined (45). An increased rate of Ag internalization is a characteristic of activated macrophages (62). If direct GaAs exposure activates macrophages, the cells may have this trait in addition to the other phenotypic changes, which could also contribute to the enhanced APC function.

GaAs exposure apparently displays the paradoxical effects of local inflammatory reactions and systemic immunosuppression. The disparate consequences of GaAs on macrophages may possibly be explained by the location of the cells relative to the exposure site and the chemical form(s) of GaAs to which the cells are exposed. A high proportion of peritoneal macrophages contains GaAs particles, whereas no GaAs crystals are detected in the spleen. Inhibitory effects of GaAs may be mediated mainly by its components, whereas direct exposure of macrophages to GaAs may activate these cells. We propose that the mode of action of the chemical on Ag processing is predominantly at the level of cathepsin activities. Based on animal studies, GaAs is classified as an immunotoxicant due to its immunosuppressive effects (63). Studies concerning industrial workers have emphasized monitoring exposure levels (64–66), rather than assessing immunocompetency. Thus, the actual health risk of occupational exposure of workers to GaAs remains unclear (64–66), and recent advances in GaAs transistor production (67) may lead to increased use of GaAs in the electronics industry. Understanding of the mechanisms by which GaAs and its components alter immune functions may provide insights into circumventing the effects of the chemical on the immune system.

Acknowledgments

We thank Dr. Albert Munson of the National Institute of Occupational Health and Safety (Morgantown, WV) for providing gallium arsenide and helpful discussions.

References


