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J Immunol 2000; 165:750-759; doi: 10.4049/jimmunol.165.2.750
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Involvement of an ATP-Dependent Peptide Chaperone in Cross-Presentation After DNA Immunization

Udayasankar Kumaraguru,* Richard J. D. Rouse,† Smita K. Nair,‡ Barry D. Bruce,§ and Barry T. Rouse2*

Immunization with plasmid DNA holds promise as a vaccination strategy perhaps useful in situations that currently lack vaccines, since the major means of immune induction may differ from more conventional approach. In the present study, we demonstrate that exposure of macrophages to plasmid DNA encoding viral proteins or OVA generates Ag-specific material that, when presented in vitro by dendritic cells to naive T cells, induces primary CTL response or elicits IL-2 production from an OVA peptide-specific T-T hybridoma. The immunogenic material released was proteinaceous in nature, free of apoptotic bodies, and had an apparent m.w. much larger than a 9–11-aa CTL-recognizable peptide. The macrophage-released factor(s) specifically required a hydrolyzable ATP substrate and was inhibited by procedures that removed or hydrolyzed ATP; in addition, antihemagglutinin protein 70 antiserum abrogated the activity to a large extent. These results indicate the possible involvement of a heat-shock protein 70-linked peptide chaperone in a cross-priming method of immune induction by DNA vaccination. Such a cross-priming process may represent a principal mechanism by which plasmid DNA delivered to cells such as myocytes effectively shuttle Ag to DC or other APC to achieve CTL induction in vivo. The Journal of Immunology, 2000, 165: 750–759.
Materials and Methods

Mice

Seven- to eight-week-old and retired breeder female C57BL/6 mice (H-2b) and BALB/c mice (H-2d) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). In conducting the research described in this work, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Cell line and cell culture

The tumor cell lines used were BALB/3T3 (BALB/c, H-2d fibroblast), EL4 (C57BL/6, H-2d lymphoma), EG7 OVA cells (EL4 cells transfected with the cDNA of chicken OVA) (17), EMT6 (BALB/c mammary adenocarcinoma cells, H-2d; kindly provided by Dr. Ed Cantin, City of Hope National Medical Center, Duarte, CA), CH.2.B-1 (H-2b), B cell lymphoma kindly provided by Daniel Muller, University of North Carolina, Chapel Hill, NC), and YAC-1 (mouse lymphoma target for NK cells). All cell lines were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Life Technologies). Target cells expressing HSV proteins, EMT6-gB, EL4-gB, BALB/3T3-gB, or ICP27 were generated by transfecting the cells overnight with DNA encoding the corresponding Ags (described under cytotoxicity assay). RF33.70 (T-lymphoma) was obtained from Dr. Kenneth L. Rock (Dana-Farber Cancer Institute, Boston, MA) and grown in 10% RPMI 1640.

Peptides and plasmid DNA-encoded Ags

The H-2d-specific HSV peptide (aa 498–505: SIEFARL) and chicken OVA peptide (aa 257–264; SIINFEKL) were synthesized and deubiquitinated to provide free amino and carboxyl ends (Research Genetics, Birmingham, AL). cDNA encoding the HSV-1 genes gB and ICP27 was inserted into the pcDNAI vector (Invitrogen, San Diego, CA), as described earlier (6). pcDNA encoding chicken OVA was obtained from Dr. Michael Bevan (University of Washington, Seattle, WA) (17). The purity and concentration of plasmid DNA, which were produced LPS-free using endo-free columns (Boehringer Mannheim Biochemicals, Indianapolis, IN), were analyzed by measurement of absorbance at 260 and 280 nm and by agarose gel electrophoresis and ethidium bromide staining. Expression of the gB, ICP27, and OVA gene products was confirmed by Western blot analysis using endo-free columns (Boehringer Mannheim; catalog number G8877), and UTP (Sigma; catalog number U6625). The agents for the enzymatic treatment included a general protease-thermolysin (Boehringer Mannheim Biochemicals, Indianapolis, IN). Plasmid pcGREEN LANTERN-1 (catalog number 10642-015) was purchased from Life Technologies.

Biochemical reagents

ATP (Sigma, St. Louis MO; catalogue number A9187), adenosine 5’-O-(3-thiotriphosphate) (ATP-S; Sigma; catalogue number A1388), CTP (Acros Organics, Piscataway, NJ; catalogue number 3012-37-5), GTP (Sigma; catalogue number G8877), and UTP (Sigma; catalog number U6625). The reagents for the enzymatic treatment included a general protease-thermolysin (Boehringer Mannheim; catalogue number 161586) (EDTA was used in parallel to negatively regulate the thermolysin activity as a control), apyrase (Sigma; catalogue number A6410), which is an ATP and an adenosine 5’-diphosphatase, DNase I (Sigma; catalogue number D4263), and RNase A (Sigma; catalogue number R8475), ATP agarose (Sigma; catalogue number 02065) for making columns.

Antibodies

Anti-hsp104 (StressGen, Victoria, BC, Canada; catalogue number SPA 1040), anti-gp96 (gift from Dr. P. Srivastava, University of Connecticut), anti-hsp70 directed against a highly conserved 13-aa region near the N terminus of hsp70 (StressGen; catalogue number SPA 801), and normal rabbit serum (Zymed, San Francisco, CA; catalogue number 01-6101). PE-labeled anti-CD11b (PharMingen, San Diego, CA; catalogue number 01715B) for macrophages, PE-labeled anti-CD3 (PharMingen; catalogue number 01085B) for T cells, FITC-labeled anti-B220 for B cells (PharMingen; catalogue number 0125B), PE-labeled anti-CD11c (PharMingen; catalogue number 09705B), PE-labeled anti-CD80 (PharMingen; catalogue number 09605B), PE-labeled anti-CD86 (PharMingen; catalogue number 09275B), and FITC-labeled anti-H-2d (PharMingen; catalogue number 06044D) for dendritic cells were used.

Isolation and purity of APC and responder T cells

To isolate DCs, splenocytes were obtained as per the procedure mentioned elsewhere (19). Briefly, splenocytes were obtained from naïve mice, and the cell concentration is adjusted to 2 × 10⁶ cells in 3 ml of RPMI 1640 medium containing 10% FCS (RPMI-10% FCS). These cells were overlaid onto 2 ml of 14.5% metrizamide gradient column. After a low speed centrifugation (200 x g for 10 min), cells from the interface were collected and washed twice in RPMI-10% FCS. The pellet was resuspended in another 3 ml of the same medium and the above procedure was repeated. Cells from the interface were collected. The purity of the preparation was checked by surface staining with mAbs to 33D1 (kindly provided by Dr. Ralph Steinman, The Rockefeller University, New York, NY) and PE-labeled CD11c. The maturation status of the DCs, as analyzed by staining for MHC class II and co-stimulatory molecule (CD80 & CD86) expression, indicated it to be of heterogenous population. The Mo6 were isolated as per method described previously (19). Briefly, the splenic cells were allowed to adhere onto a plastic tissue culture T-150 flask for 90 min at 37°C. The nonadherent cells were processed for isolation of T cells described elsewhere. The adherent cell population was scraped off and allowed to readhere for another 1 h at 37°C. The readhered population was dislodged and resuspended in RPMI 1640 with 5% FBS. Flow-cytometric analysis was done after staining with mAb to CD11b to check for purity.

T cell isolation was done by separating B cells from the nonadherent population from above by passing through a nylon wool column and subsequently panning on anti-Ig-coated plates. The separated cell population was analyzed for percentage of T lymphocytes by FACS analysis (anti-CD3 staining) and later used as responder naïve T cells.

Flow-cytometric analysis

A portion of the isolated splenic populations was analyzed for cell surface markers by flow cytometry to assess the purity of the preparations. The cells were blocked with heat-inactivated FBS and washed three times with FACS buffer (1 × PBS with 1% BSA and 0.05% NaN₃). The cells were stained with mAb to 33D1, CD11c for DCs, PE-labeled anti-CD11b for Møs, PE-labeled anti-CD3 for T cells, and FITC-labeled anti-B220 for B cells.

In vitro CTL induction

T cells (5 × 10⁶ cells/ml) and APC (5 × 10⁵ cells/ml) were cultured in 100 μl of NCTC 109 and RPMI 1640 (1:1 v/v; Life Technologies), supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM oxaloacetic acid, 0.2 U/ml bovine insulin, and 5 x 10⁻³ M 2-ME in 96-well U-bottom plates to give a responder:stimulator (R:S) ratio of 10:1. After 5 days, the cells were used as effectors in a standard 4-h ⁵¹Cr release assay.

Møs (5 × 10⁶ cells/ml) were treated with purified pcDNAIgB or pcDNAICP27 (5–7 μg/ml) for 24 h in 96-well flat-bottom plates at 37°C. Following incubation for 24 h at 37°C and 5% CO₂, 100 μl of the supernatant was harvested, passed through a 0.45-μm filter, and added to 100 μl of DC-T cell microculture (R:S ratio of 10:1) in 96-well U-bottom plates. Cultures were incubated at 37°C for 5 days, following which the cells were pooled and used as effectors in a standard 4-h ³²Cr release assay.

Cytotoxicity assay

Target cells (5–10 × 10⁶ cells) were incubated with pcDNAIgB or pcDNAICP27 (100 μg) in the presence of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (50 μg) in Opti-MEM using standard transfection procedures for 12–15 h at 37°C to generate cells expressing gB or ICP27, followed by labeling with 100 μCi of ³²Cr. For peptide-specific lysis, 2 × 10⁴ target cells were labeled in 500 μl RPMI 1640 with 100 μCi of ³²Cr for 90 min with appropriate peptides at a concentration of 7.5 × 10⁻¹⁰ M. For OVA-specific lysis, 2 × 10⁵ ⁷⁵lCr were pulsed for 90 min with 100 μCi of ³²Cr in 500 μl of RPMI 1640. After washing, 10⁴ labeled targets and serial dilutions of effector cells were incubated in 200 μl of RPMI 1640 with 10% heat-inactivated FCS in 96-well V-bottom plates. The plates were centrifuged at 500 x g for 3 min and incubated at 37°C and 5% CO₂ for 4 h. A total of 100 μl of the supernatant fluid was collected, and radioactivity was measured using a LKB gamma counter, and specific cytotoxicity activity was determined using the formula: % specific release = [(experimental release – spontaneous)/(total release – spontaneous release)] × 100, where experimental release is the radioactivity present in test samples, while spontaneous release is the radioactivity of targets with the addition of media only and the total release is the measure of activity after the addition of 3% Triton-X. Each assay was performed in triplicate, and the spontaneous release was less than 22% of total release by detergent in all assays.
Table I. OVA-specific CTL induction in vitro by transfection or pulsing of DC with plasmid DNA encoding OVA.

<table>
<thead>
<tr>
<th></th>
<th>LU per Culture</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA DNA transfected DC</td>
<td>58</td>
<td>69</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>OVA DNA pulsed, 5 μg/ml</td>
<td>&lt;18</td>
<td>&lt;12</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>OVA DNA pulsed, 40 μg/ml</td>
<td>18</td>
<td>12</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*a* DCs were either transfected or pulsed with OVA DNA and mixed with naive T cells for primary in vitro CTL generation. The effectors were mixed with OVA-specific targets (EG7) at varying ratio in a 51 Cr release assay. Data are expressed as LU, where 1 LU is the number of lymphocytes required to yield a lysis value of 20%.

Calculation of lytic units (LU)

A plot with percentage specific lysis value vs the log of the effector cell number for each effector cell preparation was done. A lysis value of 20% was selected through which most of the declining titration curves passed. One LU was the number of lymphocytes required to yield 20% lysis. If the titration curve of an effector cell preparation failed to reach the selected lysis value (20%), the activity was referred to as <x> LU, where “x” is the calculated minimum level.

**Induction of IL-2 in DC-RF33.70 cells**

The RF33.70 generated by Rock (20) recognizes the OVA epitope SIINFEKL presented on H-2b APC and produces IL-2 in response. The macropage-released factor (MRF) collected from the Mφ cultures treated with OVA DNA were serially diluted in a 96-well plate; to this, DC and RF33.70 cells at a ratio of 1:2 (DC, 5 × 10^5 cells/ml; RF33.70, 1 × 10^5 cells/ml) were added and incubated for 36 h at 37°C and 5% CO₂. The supernatants from these cultures were analyzed for the presence of IL-2 using CTLL-2 cells obtained from Dr. Cynthia Watson (National Institutes of Health, Bethesda, MD). The detail of the procedure for the CTLL-2 assay, including the construction of a standard curve with rIL-2, was described previously (21).

**Ultrafiltration**

The MRF were passed over a series of Centricon concentrators (Amicon, Beverly, MA) beginning with Centricon-100, in which molecules greater than 100 kDa were retained. The retentate was saved diluted to the original volume. Each retentate and the flow-through was spun over a Centricon-30. Again the retentate, macromolecules larger than 30 kDa, was saved, diluted to the original volume, and the flow-through was spun over a Centricon-10. Finally, the flow-through from Centricon-10 was passed over Centricon-3 after diluting to the original volume. Each retentate and the flow-through from Centricon were tested by pulsing naive DC and tested for primary CTL induction in naive T cells.

**Dialysis**

The MRF was injected into Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL; catalog number 66332CW), as per the manufacturer’s instruction, and dialyzed against PBS for 24 h at 4°C. The MRF were then analyzed for its potency to induce IL-2 either alone or with the addition of graded dose of ATP starting from 0.31 mM to 10 mM concentration.

**Blocking with anti-hsp Abs**

The individual fractions of MRF were treated with polyclonal Abs to hsp104, gp96, hsp70, and hsp25 at a final concentration of 1 in 200 and incubated in a 37°C waterbath for 45–60 min. As a control for this experiment, the DC and RF33.70 hybridoma were also treated with ATP (2 mM) for 30 min at 37°C, washed by passing through a Histopaque (Sigma) column, and centrifuged twice and resuspended in 10% RPMI 1640 to analyze the effect of ATP on DCs directly, and a portion of it was mixed with RF33.70 cells and treated with MRF.

**Western blot analysis**

Different dilutions of the MRF were boiled in reducing SDS-PAGE sample buffer, run on 10–20% gradient SDS-PAGE. They were then electroblotted.
to polyvinyl difluoride membrane, and Western blots were performed with Abs to hsp70, hsp90, gp96 OVA, and hsp104. Secondary Abs were conjugated to alkaline phosphatase, and Luminolpos was used for detection of bands (enhanced chemiluminescent system of detection) by exposing the membrane to a hyper film (Amersham, Arlington Heights, IL) and later processed using Konica automatic film processor.

ATP agarose column

A 2-ml column was packed with 140 mg of ATP agarose and rinsed with 15 ml of PBS. The MRF was passed through the column at the rate of 1 ml every 5 min. The filtrate was collected and frozen, to be used later. The column was rinsed again with 15 ml PBS before elution. A solution containing 10 mM ATP in PBS was used to elute the bound hsp. The eluate was collected as 0.5-ml volume with fraction collector. Each fraction was analyzed for the presence of hsp70 by running it on a 10–20% gradient gel. The gel was fixed with ethanol:acetic acid:Milli Q water (40:10:50) and processed for silver staining, as per the procedure of Maniatis et al. (22)

Results

In vitro induction of primary CD8+ T cell responses

In previous studies, we demonstrated that transfection of DC with plasmid DNA encoding gB or ICP27 of HSV caused a potent HSV-specific CTL response by naive purified T cells (6). However, without the transfecting agent, the addition of naked plasmid DNA to DC-T cell cultures induced barely detectable CTL reactivity (6). Table I shows a similar pattern of results obtained in DC-T cell cultures stimulated with plasmid DNA encoding OVA. Thus, whereas transfection of DC with OVA DNA induced specific cytotoxicity, when the plasmid was added to DC-T cell cultures without transfection, responses were trivial unless the DC-T cell cultures were exposed to high amounts of DNA (40 μg/ml or more). With Mφ as APC, primary CTL responses were induced upon exposure to 5 μg/ml of OVA DNA, but responses were less than observed when the APC were transfected DC (data not shown). Additionally, it was shown using DNA encoding the green fluorescent protein (GFP) that whereas Mφ took up and expressed the plasmid following exposure to it, GFP expression only occurred in MRF as APC supported indifferent in vitro primary CTL responses, exposure of such cells for 24 h to naked DNA (5 μg/ml) encoding HSV or OVA proteins induced the production of material (which is referred to as MRF), which, when added to naive

Table II. Effect of ATP on primary in vitro OVA-specific CTL generation

<table>
<thead>
<tr>
<th></th>
<th>MRF</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA DNA treated</td>
<td>53</td>
<td>61</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Vector DNA treated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>OVA DNA + ATP treated</td>
<td>83</td>
<td>94</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

*The MRF obtained from OVA DNA-pulsed Mφ culture was treated with ATP (2 mM, an optimum concentration based on the dose response curve obtained from the experiment using 10 mM ATP double diluted up to 0.31 mM) as described in Materials and Methods and then added to DC-T cell culture. After 5 days of incubation, cells were measured for CTL activity against EG7 (H-2b OVA), EL4 (H-2b), or EMT6 (H-2d) pulsed with SIINFEKL peptide. The control for the MRF included MRF without ATP and vector DNA-pulsed Mφ supernatant. The results are expressed as LU, wherein 1 LU is the number of lymphocytes required to give 20% lysis.
DC-T cell cultures, generated Ag-specific CTL. For example, the experiment shown in Fig. 2 indicates that when H-2d Mφ were treated with either pcDNAgB, pcDNAICP27, or control DNA-expressing luciferase (pcDNAlux), the material generated CTL in DC-T cell cultures that were specific to the protein encoded by the DNA. For example, MRF from pcDNAICP27-treated Mφ generated CTL, which lysed H-2d ICP27 targets, but not H-2d gB-expressing or mock-transfected targets (Fig. 2 and data not shown). Similarly, pcDNAgB induced MRF-generated gB-specific CTL. The pcDNAlux MRF failed to generate any Ag-specific CTL. Once again, Mφ-pcDNAgB MRF added to syngeneic H-2b DC-T cell cultures generated CTL that lysed H-2b gB targets, but not H-2b ICP27 targets (see Fig. 3). The OVA DNA CTL induction data are shown in Table II and discussed subsequently.

Identity of immunogenic material generated by Mφ

Several possibilities were considered as to the identity of the immunogenic material released from the DNA-treated Mφ. The idea that the MRF might represent free peptide was made unlikely by the data presented in Fig. 4, in which the MRF were passed through ultrafiltration filters of various sizes before assay for CTL induction. As is evident, the maximum CTL activity was generated with fractions that were 30–100 kDa. Fractions that were >100 kDa and 10–30 kDa generated some activity, but fractions less than 10 kDa that would include peptides of 9–11 aa were without activity.

To further facilitate characterization of the MRF, an assay that employed an OVA peptide-specific CD8+ T cell hybridoma (RF33.70) was used. In addition, H-2d Mφ exposed to OVA DNA was the production system employed. As is shown in Fig. 5A, the OVA DNA-induced MRF that effectively generated primary
FIGURE 6. Characterization of the nature of MRF by enzyme digestion and centrifugation. The MRF generated from H-2^b Mφ with pcDNAOVA were subjected to high speed centrifugation at 117,000 × g for 1 h or treated with various enzymes (as shown) and incubated for 1 h at 37°C before adding to DC-RF33.70 cell cultures. The supernatant from this culture was harvested after 36 h for IL-2 quantification by the CTLL-2 bioassay. Data are represented as IL-2 pg/ml. Figure shows the mean of three separate experiments.

OVA-specific CTL in DC-T cell cultures also induced the production of IL-2 from DC-RF33.70 hybridoma cultures (Fig. 5B). The latter cultures also responded to stimulation by the synthetic peptide SIINFEKL, although the presence of DC to present the peptide was necessary for this to occur. Similar concentrations (2–10 μg/ml) of peptide failed to induce primary OVA-specific CTL responses in DC-T cell cultures. The DC-hybridoma system failed to respond to MRF from vector DNA-treated Mφ and failed to react to OVA DNA unless exposed to high concentrations (40 μg/ml or more). Because the active MRF were generated at a DNA concentration of 5 μg/ml, most of which was taken up or degraded, residual DNA (max levels of 0.7 μg/ml were detected) would not appear to account for the IL-2-inducing effect.

The nature of the inducing factors in the MRF was analyzed by subjecting it to enzyme digestion before titration in the DC-RF hybridoma test system. In addition, high speed centrifugation (117,000 × g for 60 min) was used in some experiments to determine whether the active material was particulate, such as apoptotic bodies released from dying Mφ. Centrifugation had no measurable effect on the activity of MRF (Fig. 6). In addition, the MRF appeared to be neither RNA nor DNA because treatment with RNase and DNase failed to diminish the activity (Fig. 6). However, treatment with the general protease thermolysin (metalloendopeptidase) almost completely eliminated the activity, an effect neutralized by chelating with EDTA known to prevent the action of thermolysin (23) (Fig. 6).

A likely explanation for the identity of the MRF following exposure to DNA was a chaperone-bound peptide such as has been advocated to explain cross-priming in some tumor systems (24). Because the function of several chaperones is affected by ATP (25), the effect of ATP and other nucleotide triphosphates on the activity of MRF was measured. It is clear from Fig. 7 that treatment with ATP, but none of the other triphosphates, enhanced the activity of MRF. This ATP-enhancing effect was largely counteracted by pretreatment with a molar excess of the competitive inhibitor ATP-γ-S. Whereas 2 mM ATP enhanced the activity of the MRF, ATP failed to enhance the IL-2 induction effect of any concentration of the peptide SIINFEKL (Fig. 7). This further argues that the activity being detected in the MRF was unlikely to be free peptide. In other experiments, the effect of 2 mM ATP addition on the magnitude of the OVA-specific CTL induction response was investigated. The presence of ATP in supernatants enhanced the CTL induction response by about 25% (Table 2). In addition, treatment of DC with 2 mM ATP, followed by cell washing before their exposure, failed to influence the response significantly (Fig. 7). Thus, the effect of ATP may not be mediated by nonspecific activation of DC or RF33.70.

In other experiments, the active MRF was dialyzed for 24 h in an attempt to remove any ATP present that could confuse the interpretation of the enhancement effect of exogenously added ATP. As is shown in Fig. 8, dialysis reduced the activity, although not to baseline, but the addition of 2 mM ATP enhanced the activity to an even greater extent (up to 6-fold) than the ATP-mediated enhancement observed with nondialyzed MRF (up to 1.9-fold).
Dialysis of MRF and effect of addition of exogenous ATP. The MRF obtained from H-2b Mφ pulsed with pcDNAOVA was dialyzed for 24 h against PBS at 4°C to remove the endogenous ATP and then analyzed for their IL-2 induction ability in the DC-RF33.70 culture. A portion of the dialyzed MRF was treated with different concentrations of ATP before adding to the DC-RF33.70 cultures to analyze the effect of addition of exogenous ATP. The experiment of similar design was performed three times, and the data show the mean of all three.

The presence of hsp70 in the MRF preparation was also shown directly by purifying the MRF and by Western blot. Thus, the eluate from the MRF that was subjected to purification of the hsp70 by using the ATP agarose column yielded a positive band by silver staining procedure. This direct evidence implicates a possible role for hsp70 (Fig. 9A). Western blot probing for the presence of hsp and OVA protein was also additionally performed and probed with Abs to OVA, hsp104, gp96, and hsp70. A highly sensitive chemiluminescent detection system showed a prominent hsp70 band (Fig. 9B) as well as a very weak band for hsp104 and gp96 (however, our attempts to procure standard proteins were only successful with hsp70 and OVA). The anti-OVA Ab failed to show a 45-kDa band that is indicative of intact OVA protein.

Further approaches were used to implicate a likely role for hsp70 as responsible for the activity of MRF. First, as shown in Fig. 9, by silver staining and western blot analysis, hsp70 was readily demonstrable in the MRF (Fig. 9A). Other chaperones such as hsp90 (Fig. 9B), gp96, or hsp104 (data not shown) could not be demonstrated. Soluble OVA protein was also not found in the MRF. Purification of the MRF on ATP agarose column, followed by SDS-PAGE and silver staining, revealed the presence of a 70-kDa but not 90-kDa protein.

Second, a role for hsp70 was analyzed by measuring the effects of anti-hsp70 and other anti-chaperone proteins on the function of MRF measured by the DC-hybridoma assay. As is evident in Fig. 10, the function of MRF was inhibited by polyclonal anti-hsp70 Abs, but only marginally inhibited by other anti-chaperone Abs. The anti-hsp70 serum appeared to have no effect on either the DC or RF33.70 cells used in the assay (Fig. 10).

**Discussion**

Immunization with plasmid DNA holds promise as a strategy to protect against certain agents that currently lack effective vaccines. Such genetic vaccines may overcome the shortcomings of more traditional approaches because the principal methods by which DNA vaccines induce immunity may be unusual. In the present study, our data demonstrate that exposure of Mφ to plasmid DNA encoding viral proteins generates Ag-specific material that, when presented in vitro by mature DC to naive T cells, induces primary CTL responses. Administration of plasmid DNA to the same system failed to induce CTL presumably because mature DC take up and express the DNA poorly (6). The immunogenic material released was proteinaceous in nature, free of apoptotic bodies, and had an apparent m.w. much larger than a 9–11-aa CTL-recognizable peptide. The cross-presentation process specifically required hydrolyzable ATP and was inhibited by procedures that removed or hydrolyzed ATP. These properties plus the ability of anti-hsp70 antiserum to partially abrogate the activity indicated the possible involvement of a hsp70-linked peptide chaperone. This cross-presentation mechanism of immune induction may represent a principal method by which plasmid DNA delivered to cells such as myocytes effectively shuttle Ag to DC or other APC to achieve CTL induction in vivo.

Upon introduction as a vaccination strategy, i.m. injection was the favored route for DNA immunization (8). Gene expression was readily evident in myocytes, but such cells were not expected to act as APC for immune induction. A more likely alternative is that rare
bone marrow-derived cells in muscles, or cells of bone marrow origin transfected by plasmid DNA in the bloodstream, or lymphoid tissue act as the APC for immune induction (5, 8). Recent reports confirm the essential role of bone marrow-derived cells rather than myocytes for CTL induction (3, 4), and transfected DC have been observed in recipients of DNA vaccines (5, 26–28). Consequently, immune induction following DNA vaccination could be driven principally by APC, which themselves are transfected by plasmid DNA. Although DC and other APC may contain plasmid DNA in vivo, DC, especially in their mature Ag-presenting stage, take up plasmid DNA ineffectively and in fact may sequester the DNA in endosomes that would prevent expression (29). Accordingly, alternative means of acquiring Ag for immune induction may operate. Our data provide evidence that the cross-priming process initially described by Bevan (17) represents one such mechanism. In our experiments, the cross-presentation material was produced by Mφ exposed in vitro to plasmid DNA encoding either viral Ags or OVA. Whereas Mφ readily took up plasmid DNA, this was not the case for splenic DC. Only when exposed to high concentrations of plasmid DNA (40 μg/ml) did DC-T cell cultures respond. In contrast, Mφ, although poor APC in the primary antiviral system (6), rapidly produced the cross-presentation material upon exposure to low levels of plasmid DNA (5 μg/ml or less).

The types of events involved in cross-priming were succinctly reviewed by Bevan (14), although this review only dealt with responses to peptide/protein Ags. Our novel studies indicate that a cross-priming process can also occur during immune response to DNA vaccines, and that the process may involve an ATP-dependent chaperone protein such as hsp70 bound to the CD8+ T cell-recognized peptide. Thus, the MRF activity was protease sensitive, yet was resistant to both RNA and DNA nucleases. The MRF was unlikely to include apoptotic bodies as described by Bhardwaj and colleagues (16) as a means of Ag transfer between cells, nor could it simply be a MHC-binding small peptide. Accordingly, the activity was unaffected by high speed centrifugation and in being largely between 30 and 100 kDa in size, not a 9- to 11-aa peptide. The size profile could implicate peptides associated with either members of the hsp70 or hsp90 class of molecular chaperones (25). Furthermore, our data clearly showed that the addition of exogenous ATP enhanced the activity of MRF, as measured both in the CTL induction assay and an assay in which the material was presented by DC to an IL-2-producing T cell hybridoma. In fact, the stimulatory effect of exogenous ATP was even more marked when the MRF was dialyzed before assay to remove the endogenous ATP initially present. In this instance, the activity, at least as measured in the T cell hybridoma assay, was enhanced by ATP up to 6-fold. In contrast, the presence of exogenous ATP failed to influence the response of DC-hybridoma cultures to free peptide, and ATP treatment of DC in the absence of MRF failed to change their APC activity.

The pronounced effect of treatments that alter the levels of ATP suggests that the MRF requires ATP binding to function. In addition, the ability of ATP-γ-S to inhibit this activity indicates that ATP hydrolysis is involved during MRF function. These properties...
are consistent with the involvement of a class of molecular chaperone whose function is influenced by ATP. The most likely candidates are members of the hsp70 class of molecular chaperones that contain both an ATP binding domain at the N terminus and a peptide domain at the C terminus (30, 31). The two domains interact, and one consequence of ATP binding is the release of peptide from its binding domain (32, 33). The function of other chaperones such as hsp90 may also be influenced by ATP, although in the case of hsp90, the reported data are conflicting (34–36), and hsp90 appears to lack known ATP-binding motifs (37).

The presence of a prominent hsp70 band and the absence of OVA protein, as evidenced by Western blot analysis, strongly implicate hsp70 chaperone in the process and negate a role for the soluble OVA protein. An additional experiment also supported a likely role for an hsp70 as principally involved in the cross-presentation activity of MRF. Thus, the addition of antisera to hsp70 markedly reduced the activity. The effect was almost unaffected by anti-gp96, perhaps not surprising because this chaperone most likely functions independently of ATP (38). Nevertheless, at least in some tumor systems, in which peptide-bound chaperones can be isolated from cells and shown to be immunogenic, gp96 as well as other chaperones such as calreticulin can be involved as carriers of peptides (39–41). The residual activity detected in our experiments after anti-hsp70 treatment indicates the likely involvement of other classes of molecular chaperones in the cross-presentation process. Currently, we fail to understand the mechanism by which the chaperone-bound peptides are generated within Mφ and released to the outside. This issue is under further investigation.

It remains to be shown how the DNA-induced ATP-dependent chaperone-bound material transfers immunogenicity to DC, as well as to be established whether similar events also occur in vivo, perhaps involving myocytes and DC. With regard to the former issue, ATP activation is known to cause peptide release from chaperones that contain ATP binding domains (42). Conceivably, therefore, the increase in activity observed following the addition of ATP could represent a cell surface event with the released peptide exchanging with material bound to MHC class 1 on DC. However, due to the limited concentration of these peptides, a facilitated exchange/delivery system would most likely need to occur. Alternatively, the ATP-dependent chaperone could be internalized effectively following receptor binding to the surface of DC. This would need to be followed by a peptide exchange between the chaperone and newly formed MHC class I proteins. It is not clear if or where such exchanges occur in the cell. Clearly, additional experiments are required to define mechanisms by which ATP influences the cross-presentation process.

Finally, our results emphasize the importance of cross-priming as a mechanism by which DNA vaccines might achieve immunogenicity. If the process we describe is an important component of antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. J. Exp. Med. 186:1481.


