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Cell-Mediated DNA Transport Between Distant Inflammatory Sites Following Intradermal DNA Immunization in the Presence of Adjuvant

Antonio La Cava, Rosario Billetta, Guido Gaietta, Dustin B. Bonnin, Stephen M. Baird, and Salvatore Albani

After intradermal genetic immunization, naked DNA is transported from the site of injection to regional lymph nodes. Little is known on how inflammation influences this process and whether DNA is transported beyond local lymph nodes. In the experiments herein reported, we injected naked DNA in the presence of adjuvant to address questions related to 1) the fate of naked DNA in the presence of inflammation; 2) the generation of immune responses to the encoded protein during inflammation; and, more in general, 3) the fate of ingested molecules beyond regional lymph nodes during inflammation. Two sites of inflammation were induced in vivo in mice. Naked DNA was injected in the nape together with adjuvant, and adjuvant only was injected at a distant peritoneal site. Injected DNA, uptake at the primary dermal site of inflammation, was transported beyond regional lymph nodes to distant organs such as the spleen and to the distant peritoneal site of inflammation. This transport, mediated by CD11b+ cells, was cumulative during chronic inflammation. These results indicate a novel route of transport of DNA beyond regional lymph nodes and may have specific implications for DNA-based immune modulation. The Journal of Immunology, 2000, 164: 1340–1345.

In vivo injection of naked DNA elicits strong immune responses against the encoded Ag (1). Hence, genetic immunization has been used to generate protective humoral and cell-mediated immune responses (2, 3) in a wide variety of preclinical animal models for infectious diseases, allergy, cancer, and autoimmunity (4).

It is currently accepted that genetic immunization is based on the in situ transfection of cells whose identity varies in relation to both the method (5, 6) and the site of injection, i.e., the muscle vs the skin (7, 8). Muscle cells are transfected following i.m. injection of DNA (9, 10). Keratinocytes, fibroblasts, and dendritic cells are transfected after intradermal (i.d.)3 immunization (11–14). Bone marrow-derived APC and keratinocytes are directly transfected after gene gun immunization (15, 16). It has been suggested that the strong and persistent humoral and cellular immune responses that follow i.d. DNA immunization can be related to the important immune surveillance functions of the skin and the skin-associated lymphoid tissues (17).

Cells of the dendritic lineage such as the bone marrow-derived Langerhans cells (LC) constitute about 5% of epidermal cells. Along with skin macrophages, LC are specialized in uptaking foreign Ags for transport to draining lymph nodes, where primary immune responses are initiated (18, 19). LC efficiently phagocytose both naked DNA and proteic Ags and are a major APC population in the immune response that follows i.d. genetic immunization (15, 20). Once transfected, skin-derived dendritic cells convey the uptake of Ag to draining lymph nodes (13) for elaboration of primary immune responses (21). Similarly, transfected macrophages can transport in vivo-phagocytosed Ag to sites distant from injection (22). However, it remains unclear whether DNA uptake at a site of i.d. injection is transported beyond regional lymph nodes during inflammation. Such information could have significant implications for a better knowledge of the immune responses after DNA vaccination.

In the experiments herein described, we induced two distant sites of inflammation in vivo in mice to study the effect of proinflammatory chemotactic stimuli on the transport of injected DNA-based Ag. The primary site of inflammation was produced by i.d. coinjection in the nape of adjuvant with naked DNA. The second inflammatory site was induced by injection of adjuvant only at a distant peritoneal site. By combining construct-specific PCR with in situ hybridization and FACS, it was possible to follow, at the individual cell level, active cell-mediated transport of plasmid DNA between the two inflammatory sites. In situ hybridization and immunofluorescence studies detected the presence of plasmid DNA at the dermal site of inflammation for up to 6 wk after immunization and in situ expression of the encoded proteic product in cells of the epidermis and dermis. Injected DNA, uptaken at the dermal site of inflammation, could be transported beyond regional lymph nodes and detected in organs distant from the site of injection such as in the spleen. Repetitive administration at the primary inflammatory site led to accumulation of injected DNA at the distant site of inflammation. This transport was mediated by CD11b+ cells.

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3 Abbreviations used in this paper: i.d., intradermal; LC, Langerhans cells; FISH, fluorescence in situ hybridization; ampR, ampicillin resistance gene; PEC, peritoneal exudate cell.
cells and was cumulative during chronic inflammation. The implications of this pathway for genetic immunotherapy and vaccination are discussed.

Materials and Methods

Plasmid construct

A segment of 426 b.p. (position 2995–3385) of EBV Balf2 gene was amplified from genomic DNA of EBV (strain B-958) with the primers: 5'-GCCCAAGCTTGGATACCAATGGATT-3' and 5'-GGCG GATCCGGCTAGACGAGTTGCGT-3' in an Eriocamp EasyCycler (San Diego, CA). PCR conditions were: 1 min denaturation at 95°C; 1 min 30 s annealing at 49°C for the first 5 cycles, and 1 min at 52°C during the remaining 30 cycles; 2 min amplification at 72°C and 6 min for the last extension cycle. The amplified DNA fragment was subcloned between the HindIII/BamHI sites of the eukaryotic expression vector p290 (a gift of Dr. Tyler Parr, University of Southern California, Los Angeles, CA), which contains a CMV promoter upstream of the polylinker. p290 does not replicate in mammalian cells nor does integrate into their genome. Bacterial methylation of p290:Balf2 construct was assayed by digestion with HpaII and MspI restriction enzyme isoschizomers (23).

Genetic immunization

A total of 100 μg of p290:Balf2 DNA construct dissolved in sterile endotoxin-free water were mixed or not with 100 μg of the DNA-free synthetic adjuvant Adjuprime (Pierce, Rockford, IL) and injected i.d. into the nape of BALB/c mice (Jackson ImmunoResearch, West Grove, PA). Then, 100 μg of Adjuprime only were injected into the same mice i.p. Injections were performed, when required by the protocol, at weekly intervals. Sets of experiments were performed and confirmed at least three times. Intensity of fluorescence in situ hybridization (FISH) experiments were performed and confirmed at least three times.

Hematoxylin/eosin staining

Hematoxylin/eosin staining of cryosections of skin at the site of injection was performed using standard procedures, as described (24).

Fluorescence in situ hybridization (FISH)

Slides were treated with 30 μl of 100 μM proteinase K (Sigma, St. Louis, MO) for 30 min at 37°C and then fixed in formaldehyde for 5 min at 4°C. A Balf2 gene probe corresponding to positions 3144–3202 was 3'-tailled in the same way and used as a negative control. Reaction mixture was mixed or not with 100 μg of the DNase-free synthetic oligonucleotide corresponding to the positions 3144–3202 of the Balf2 gene was used as a probe. The membrane was hybridized at 42°C overnight, then washed in 2% ethidium-bromide-stained agarose gel.

PCR

Total DNA, isolated and purified from PBMC, spleen, and skin by Easy DNA kit (Invitrogen, Carlsbad, CA), was quantified by DNA Dipstick (Invitrogen). Then, 40 ng of total DNAs were used as templates for PCR with the conditions described above. A second round of PCR amplification was performed with identical conditions on one-tenth of the amount of the first PCR product.

For semiquantitative PCR, two independent PCR were performed, each on DNA from 5 × 10⁶ PBMC harvested at the second week of immunization on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. One PCR was conducted with primers for Balf2, and the other PCR with primers for the ampicillin resistance gene (amp'): 5'-GGCTCCAGGTTTAT CAGCAATAACCA-3' and 5'-ATACACTTCTTCAGAATGCTT GGT-3'. A single copy of the amp' gene is present in the plasmid construct containing the Balf2 gene. The length of Balf2 and of amp' PCR products are comparable. Starting from 25 ng, 1:2 scalar dilutions of p290 plasmid DNA were used as control templates for the PCR on amp'. Intensity of amp' bands was compared with the intensity of the Balf2:PCR product on a 2% ethidium-bromide-stained agarose gel.

Southern blotting

Total DNA was extracted with the Easy DNA kit (Invitrogen) according to the manufacturer’s instructions. Balf2-specific PCR products were blotted on Hybond N membrane (Amersham, Buckinghamshire, U.K.). A digoxi- genin-labeled synthetic oligonucleotide corresponding to the positions 3144–3202 of the Balf2 gene was used as a probe. The membrane was hybridized at 42°C overnight, then washed in 0.1% SSC/0.1% SDS, blocked for 30 min with TBS/2% casein (Sigma), and incubated at room temperature for 30 min with AP-conjugated anti-digoxigenin Ab (Boehringer Mannheim) diluted 1:1000. After washes and incubation with the nonradioactive substrate Lumi-Phos (Boehringer Mannheim), the membrane was exposed to x-ray film (Kodak, Rochester, NY).

Confocal microscopy

Cryosections of skin at the site of injection were fixed in 2% paraformaldehyde/PBS for 10 min at room temperature, permeabalized with 0.2% Triton X-100/PBS for 10 min, and then blocked with PBS/0.05% BSA (Sigma). Tissue sections were double stained with phallidin-FITC (Sigma) and anti-Balf2 rabbit antisera plus rhodamine anti-rabbit Ab (H+L Ab (Cappel ICN, Costa Mesa, CA) in 0.005% BSA. Sections were examined with a Bio-Rad MRC 1024 Laser scanning confocal system (Bio-Rad, Hercules, CA) coupled to a Zeiss Axiovert 35 M microscope (Zeiss AG, Oberkochen, Germany). Individual images (1024 × 1024 pixels) were saved to optical disk (Pinnacle Micro, Irvine, CA), converted to PICT format, and merged as pseudocolor images with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Individual-cell PCR/FISH/flow cytometry

PBMC were purified on Ficoll-Hypaque gradient (Pharmacia). Monocytes were further separated by FACS by anti-CD11b Ab (PharMingen, San Diego, CA), fixed in PBS/2% paraformaldehyde, and permeabilized with streptolysin O (Sigma) at 37°C for 30 min. Cells were pelleted and resuspended in PCR mix. PCR was performed as described above. As a positive control, primers for G3PDH were used. A digoxigenin-labeled irrelevant probe was used as a negative control. After cell pelleting and washing in 2× SSC and PBS, FISH was performed on cells in suspension. After incubation in PBS/2% BSA for 30 min at 4°C, FITC anti-digoxigenin Ab (Boehringer Mannheim) was added: 1:500 v/v for 30 min at 4°C. After washes, flow cytometry was performed on a FACScan cell sorter (Becton Dickinson, San Jose, CA).

Results

Uptake of i.d. injected DNA and in vivo expression of its encoded product

Six BALB/c mice were immunized i.d. in the nape with 100 μg of naked DNA construct (p290:Balf2) plus 100 μg of the adjuvant Adjuprime. The presence of the Balf2 gene and the expression of the encoded protein product were then monitored at the site of injection. p290:Balf2 construct was uptaken in vivo and could be visualized by FISH at the dermal site of inflammation (Fig. 1) for up to 6 wk after DNA immunization (not shown). Histological examination of the same area showed cellular patchy infiltrate of the dermis consistent with nonspecific inflammatory reaction (not shown).

In addition to the presence of plasmid DNA in the dermis at the site of injection of immunized animals, in situ expression of the encoded Balf2 proteic product was detected in the epidermis (not shown) and in the underlying dermis (Fig. 2), confirming recent similar observations by others (22, 25). In particular, confocal microscopy studies indicated intracellular expression of Balf2 protein in cells with the morphology of keratinocytes of the granular layer of epidermis and phagocytic cells of the dermis. Control sections incubated with rabbit preimmune serum stained negative for the presence of Balf2 (not shown).

DNA transport from the site of injection to distant sites

To follow the fate of the DNA molecules uptaken at the i.d. site of inflammation, total DNA was extracted from four injected animals from the skin of the neck at the site of injection, from PBMC, from spleen, and from the skin of the tail, which is a site distant from DNA injection. Balf2-specific PCR in individual mice showed the presence of injected DNA construct in all these tissues, except the skin of the tail (Fig. 3). The amount of transported plasmid construct was about 40 pg/10⁶ PBMC, as determined by semiquanti- tative PCR analysis (not shown). This transport was cell mediated, as indicated by intracellular colocalization of the construct and of...
its encoded product and by the fact that PCR on serum of immunized animals tested negative (not shown).

To rule out any potential effect of the plasmid and/or genetic construct on the DNA transport, a second set of four mice was injected with a PCR-amplified gene construct encoding for the streptococcal protein M5, and a third set of four mice with a PCR-amplified gene construct encoding the region 1495–1935 of EBV Balf2. A different eukaryotic expression vector was also employed (pRC/CMV; Invitrogen). In all such cases, PCR amplification of relevant DNA fragments revealed the presence of injected DNA in PBMC and spleen (not shown).

Cell-mediated transport of DNA between distant inflammatory sites

To address the possibility that ingested DNA molecules could be transported between two distant inflammatory sites, two simultaneous foci of inflammation were generated in the same animal of a group of six. The primary site of inflammation was induced by immunizing mice with naked DNA plus adjuvant i.d. in the neck, and a second site was induced by concomitant injection of adjuvant only at a distant peritoneal site. Peritoneal exudate cells (PEC) were collected by lavage 3 days after the third of three i.d. immunizations associated with i.p. injection of adjuvant performed at weekly intervals. Total DNA was extracted from PEC and amplified by Balf2-specific PCR. Southern blotting on PCR products with an internal Balf2 probe indicated cell-mediated transport of the DNA-based Ag from the dermal site of injection to the peritoneal site of inflammation (Fig. 4). Intradermal injection in the nape of mice with adjuvant only tested negative, whereas lack of inflammation at the distant site was associated with diminished DNA transport (Fig. 5).

Cumulative effect of chronic inflammation on the transport of DNA

To evaluate the effect of repetitive administration of naked DNA during inflammation, three groups of three mice were immunized i.d. in the neck with naked DNA construct at weekly intervals. Inflammation was induced in both neck and peritoneum by concomitant injection of adjuvant in both sites. One mouse from each
group was sacrificed 3 days after 1 wk of immunization, another mouse 3 days after 2 wk of immunization, and the last mouse 3 days after 3 wk of immunization. Total DNA was extracted from peritoneal lavage cells (site of secondary inflammation), and Balf2-specific PCR on DNA from peritoneal cells was followed by Southern blot with an internal probe for Balf2. Southern analysis showed that repeated i.d. administration of DNA at the primary inflammatory site led to its accumulation at the peritoneal inflammatory site (Fig. 4), but not at an unrelated distant site such as the skin of the tail (not shown).

**CD11b<sup>+</sup> cells mediate the DNA transport between distant inflammatory sites**

Because CD11b<sup>+</sup> cells at the primary site of inflammation associated with uptake of DNA (not shown), we tested whether CD11b<sup>+</sup> cells could transport DNA to distant sites. To this aim, we used a novel strategy that is modification of a technique described by Patterson et al. (26). Briefly, peritoneal lavage cells and PMBC were harvested from four individual DNA-immunized mice and labeled with anti-CD11b mAb. After cell sorting by flow cytometry, cells were fixed and permeabilized. Individual cells positive for the i.d. injected Ag were visualized by flow cytometry after Balf2-specific PCR-FISH (Fig. 6). This analysis, performed on both CD11b<sup>+</sup> and CD11b<sup>-</sup> cells, showed that the majority of peritoneal cells positive for the Ag were CD11b<sup>+</sup> (Fig. 6). Thus, CD11b<sup>+</sup> cells can mediate transport of ingested DNA between distant and unrelated inflammatory sites.

**Discussion**

We show herein that association of local inflammation with i.d. delivery of DNA at the same site can affect transport of DNA beyond local lymph nodes to distant inflammatory sites. Intradermal DNA immunization in the presence of adjuvant resulted in

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**FIGURE 4.** Southern blot on Balf2-PCR on total DNA from peritoneal lavage cells at the site of secondary inflammation. Lanes 2, 3, and 4 are, respectively, derived from groups of animals immunized i.d. with DNA for one, two, or three times in the presence of adjuvant. Lane 1 is a negative control of DNA from peritoneal lavage cells from animals injected with adjuvant only at both the i.d. and the i.p. sites. Quantities of total DNA were comparable for all samples, as determined by Southern blot analysis on PCR-amplified G3PDH gene (not shown).

**FIGURE 5.** Balf2-PCR on total DNA of PEC of mice receiving or not the adjuvant at the peritoneal site concomitant with the i.d. DNA injection after three immunizations. The dashed arrow points at Balf2, the plain arrow at the housekeeping G3PDH. Lane 1, Molecular weight marker; lane 2, Balf-2 PCR on PEC from mice injected with adjuvant and not DNA; lane 3, Balf2-PCR on PEC from DNA-immunized mice not receiving adjuvant at the distant site; lane 4, G3PDH-PCR on PEC of lane 3; lane 5, Balf2-PCR on PEC from genetically immunized mice receiving adjuvant at the distant site; lane 6, G3PDH-PCR on PEC of lane 5.

**FIGURE 6.** Single-cell PCR/FISH/FACS. a, The sensitivity of the method is shown. The G3PDH gene is used as a positive control. The negative control is a nonrelated, digoxigenin-labeled probe. b and c, Comparison of the positivity for plasmid DNA in peritoneal lavage cells sorted for CD11b. More CD11b<sup>-</sup> (b, 10.3% positive) than CD11b<sup>+</sup> (c, 2.1% positive) peritoneal lavage cells contain Balf2. The M1 marker for b and c is set on the fluorescence of an irrelevant digoxigenin-labeled probe.
local presence of plasmid DNA at the dermal site of inflammation and in situ expression of encoded proteic product, as similarly reported by others (12, 21). Immunofluorescence staining indicated intracellular Balf2 protein expression in cells of the dermis with the morphology of dendritic cells and in keratinocytes of the granular layer of epidermis. By coupling an inflammatory stimulus to i.d. injection of DNA, locally recruited phagocytic cells transported plasmid DNA beyond regional lymph nodes to distant sites such as the spleen. Furthermore, this cell-mediated transport of ingested DNA was also detected at a distant site of inflammation. Repeated administration of naked DNA at the primary inflammatory site led to its accumulation at the distant site of inflammation.

DNA vaccination induces broad-based, long-lasting, Ag-specific immune responses (2) and associates with in vivo transfection of phagocytic cells (10–15) that migrate to regional draining lymph nodes (21, 27, 28). Because of the capability of dendritic cells to uptake DNA and carry it to local lymph nodes, Condon et al. proposed to use these cells as vehicles for gene immunization (13). Also, Chattergoon et al. recently demonstrated the presence of transfected activated macrophages and dendritic cells in the blood and peripheral tissues of animals inoculated i.m. with DNA (22). Schubbert et al. found in mice that ingested foreign DNA passes the intestinal wall and can be found in distant organs such as the liver and the spleen (29). We extend the above information by showing that CD11b<sup>+</sup> cells can mediate the transport of plasmid DNA between distant inflammatory sites after i.d. DNA immunization. The interpretation of our findings is schematically depicted in Fig. 7. DNA molecules are uptaken in the dermis by cells recruited at the inflammatory site. As indicated by confocal microscopy experiments, the injected DNA leads to relevant Ag expression in the host cells. CD11b<sup>+</sup> cells that contain plasmid DNA migrate beyond lymph nodes into the general circulation and can reach distant organs such as the spleen or distant inflammatory sites, possibly attracted by a gradient of chemotactic stimuli (30–33).

Interesting correlations can be found between the DNA transport and the handling of proteic Ags by the immune system. Indeed, in both cases 1) bone marrow-derived cells can actively uptake the molecule; 2) direct uptake of DNA by phagocytic cells may result in presentation of the encoded product to MHC-restricted T cells and generation of Ag-specific immune responses; 3) uptake of the molecule is increased by inflammation; and 4) transport is cell mediated and amplified by inflammation at the site of transport. These similarities may suggest the possibility that this pathway of transport can be relevant to both DNA and proteic Ags.

Our data also imply the possibility to manipulate abnormal immune responses at inflammatory sites. Different Ag-targeting strategies have been devised to specifically direct the Ag to APC to promote “site-directed immunogenesis” and obtain an effective immune response to DNA vaccines (34). Also in consideration of the findings of Song et al., who reported the suppression of ongoing inflammation in arthritis after i.m. injection of a plasmid-encoding TGF-β1 (35), we can hypothesize that, by associating inflammatory stimuli with injection of naked DNA encoding for a given soluble mediator (or for an Ag such as for example a tolerogenic peptide), one might possibly obtain the transport of the relevant molecule to a distant inflammatory site and its in situ expression. Additional studies are required to address this possibility and the relevance of this pathway to chronic inflammation associated with infection.

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References


