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Biochemistry

Recombinant vaccinia virus vaccine against the human melanoma antigen p97 for use in immunotherapy

(tumor antigens/tumor immunity/monoclonal antibodies)

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ABSTRACT **We have constructed a recombinant vaccinia virus, v-p97NY, which expresses the human melanoma-associated glycoprotein p97. Immunization with v-p97NY could induce humoral and cell-mediated immunity to p97, including delayed-type hypersensitivity, in mice and in two of two monkeys (*Macaca fascicularis*). The fact that an immune response was induced also in monkeys is important because normal cells from monkeys, but not from mice, express a low level of cross-reactive p97. Mice immunized with v-p97NY rejected transplants of syngeneic mouse melanoma expressing p97. A rejection response could be detected also when immunization was started 2 days after tumor transplantation, irrespective of whether the transplanted cells grew subcutaneously or as lung metastases. Evidence was obtained that melanoma cells lacking p97 may be killed as "bystanders" at the site of an immune response to melanoma cells expressing p97.**

The use of vaccines for active immunotherapy of cancer has long attracted attention but so far has met with limited success. Various approaches have been tested, including purified antigens (1–4) and anti-idiotypic antibodies against murine (5–7) and human (8) tumor-associated antigens.

Recombinant viruses expressing tumor antigens offer an alternative approach (9, 10). Cells infected with these recombinant viruses express tumor antigens at the surface together with the host's histocompatibility antigens and immunogenic viral proteins. This situation favors the induction of cellular immunity (11), which plays a key role in tumor rejection (12). Promising results have been obtained in mice with recombinant vaccinia viruses expressing tumor virus antigens (13, 14).

In contrast to viral antigens, most human tumor-associated antigens are differentiation antigens that are not absolutely tumor-specific but are expressed at trace levels in all normal cells (15, 16). One such antigen is p97, a cell-surface glycoprotein expressed by most melanomas with trace levels in normal adult human tissue (17). Cloning of the cDNA coding for p97 (18) has allowed the construction of a recombinant vaccinia virus, v-p97NY (19). Immunization with v-p97NY induces humoral and cellular immunity against human p97 not only in mice (19) but also, as shown in this study, in monkeys, whose normal cells express trace levels of an antigen cross-reactive with p97. Moreover, immunization of mice with v-p97NY was found to cause the rejection of syngeneic mouse tumors into which p97 has been transfected. Protection was also observed against a tumor line containing both p97-expressing and p97-negative cells. This is significant in view of the antigenic heterogeneity commonly observed in human tumors (20, 21).

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MATERIALS AND METHODS

Cells. The K1735 melanoma line subclone M2 (referred to as "par" cells) was of C3H/HeN mouse origin (22). Derivatives expressing p97 were made by transfecting the M2 cells with the p97 expression plasmid pSV2p97a (23). Three transfected sublines were utilized for this study, the M2SVp97.E/F1 (or "E" line), the M2SVp97.B/F1 (or "B" line), and the M2SVp97.A (or "A" line). These lines express an average of 2×10^5, 5×10^5, and 3×10^6 p97 molecules per cell, respectively, according to experiments in which bulk preparations of cells were tested for their ability to bind radiolabeled monoclonal antibody (mAb) 96.5, which is specific for p97 (17). The cells were propagated in Dulbecco's modified Eagle's medium (GIBCO), supplemented with 10% fetal calf serum (Sterile Systems, HyClone, Logan, UT) and 100 units of penicillin and 100 μg of streptomycin per ml.

Determination of the degree of intercellular variability in the expression of p97 on the transfected melanoma lines was done by analysis on a fluorescence-activated cell sorter, FACS IV (Becton Dickinson). After incubation with mAb 96.5 at 10 μg/ml and washing, cells were incubated with goat anti-mouse antibodies conjugated with fluorescein isothiocyanate, washed again, and analyzed as described (24).

Recombinant Virus (v-p97NY). Vaccinia virus was derived from a plaque-purified virus of the Wyeth smallpox vaccine (New York City Board of Health strain). Using the method of Mackett *et al.* (25), Hu *et al.* constructed a p97 recombinant virus, v-p97NY, which contains the entire coding sequence of p97 under control of the vaccinia virus "7.5-K" promoter (19). The parental vaccinia virus stock ("v-NY") was used for control immunization.

***In Vivo* Experiments in Mice.** Female C3H/HeN mice (Charles River Breeding Laboratories), 6–8 weeks old, were used. *In vitro* propagated tumor cells were briefly trypsinized, washed, and suspended in phosphate-buffered saline; aliquots of 100 μl were injected into mice by the tail vein to obtain lung metastases or subcutaneously to obtain solid tumor nodules. For a few experiments (see below), tumor cells were injected into the footpads of mice in a volume of 20 μl. Mice were immunized with 10 μl per animal of crude lysate containing 2×10^6 plaque-forming units (pfu) of either v-p97NY or v-NY. All immunizations were performed by tail scarification.

The appearance of lung metastases was quantitated 2 weeks after injection of tumor cells into the tail vein. This was done by intratracheal injection of India ink (15% in phosphate-buffered saline) before removal of the lungs, allowing the unstained metastases to be seen against black

Abbreviations: mAb, monoclonal antibody; DTH, delayed-type hypersensitivity; FACS, fluorescence-activated cell sorter; pfu, plaque-forming units; AbDCC, antibody-dependent cellular cytotoxicity.

normal lung tissue. The metastases were enumerated by using a dissecting microscope, and the data were presented as the total number of metastases per mouse. Experiments also were conducted to measure survival of mice challenged via the tail vein. Subcutaneously injected tumors were measured in two dimensions, mean tumor diameters were calculated, and the data were expressed as the percentage of mice with mean tumor diameters larger than 1.0 cm at the indicated number of days after challenge.

Studies also were performed to measure the outgrowth of tumor cell mixtures in v-p97NY-immunized mice. Two weeks after immunization, animals received 10^5 p97-negative "par" tumor cells in the left footpad and a mixture of 10^5 par cells and 2×10^5 p97-positive A-line cells in the right footpad; pilot studies had shown that the A cells cannot grow in these mice, probably because the cells express high levels of p97, although the A cells can grow in nude mice (C.D.E., unpublished data). The average thickness of the hind footpad from uninjected mice was subtracted from that of the tumor cell-injected animals to calculate the degree of footpad swelling associated with tumor outgrowth.

Immunization of Monkeys. Three adult male monkeys (*Macaca fascicularis*) were used. They were maintained by the University of Washington Primate Center. The monkeys were immunized three times at 1-month intervals by skin scarification with 100 μl of crude lysate containing 2×10^8 pfu of either v-p97NY (two animals) or v-NY (one animal).

Measurement of Antibody Titers. Anti-p97 antibody was quantitated by an enzyme-linked immunosorbent assay (ELISA). Immulon II ELISA plates (Dynatech Laboratories, Alexandria, VA) were coated overnight with 50 μl of purified p97 at 10 μg/ml in phosphate-buffered saline. Excess antigen was removed, and the plates were blocked, dried, and stored at room temperature until used. The plates were incubated with sera or mAb standards, washed, and treated with horseradish peroxidase-conjugated protein A (Zymed Laboratories, Burlingame, CA) followed by *o*-phenyldiamine (Zymed). Absorbance was read at 490 nm, and a standard curve was constructed from the mAb readings. Experimental samples were converted to binding equivalents of mAb by using the linear range of the standard curve (from 4 to 100 ng of mAb equivalent per ml); a unit is defined as the binding equivalent of 1 μg of mAb per ml.

Lymphoproliferative Assay. Peripheral blood lymphocytes from heparinized 5-week bleeds of monkeys were isolated on Ficoll/Hypaque lymphocyte separation medium (Organon). Lymphocytes were cultured at 1×10^5 cells per well in 0.2 ml of RPMI 1640 (GIBCO) supplemented with 10% heat-activated normal human serum in round-bottom 96-well plates (Corning) for 6 days with the appropriate antigen. Cells were then labeled for 6 hr with 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (New England Nuclear) and harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA), and the incorporated radioactivity was measured with Optifluor (Packard Instrument, Downers Grove, IL) in a Beckman LS 3801 counter. The results from quadruplicate wells were averaged. Proliferation indices were calculated by dividing the average cpm in cells stimulated with antigen by the average cpm in nonstimulated cells.

Antibody-Dependent Cellular Cytotoxicity (AbDCC). AbDCC with monkey serum (diluted 1:10) was assayed as described (26) on cells from the p97-positive human melanoma line M-2669; human lymphocytes were used as effector cells.

Delayed-Type Hypersensitivity (DTH) Testing in Monkeys. Skin testing was done 10 weeks after the initial immunization by injecting purified p97 (ranging from 0.4 to 40 μg) in 0.1 ml of phosphate-buffered saline intradermally on the shaved front side of the trunk. As a positive control, we gave 10^7 pfu of inactivated v-NY lysate. Swelling and erythema were monitored 48 hr later. The DTH nature of the reaction was confirmed by histopathological examination.

RESULTS AND DISCUSSION

Protection of Mice Against Intravenous Tumor Challenge. Hu *et al.* have reported that immunization of mice with v-p97NY induces both antibody formation and helper T-cell response to p97 (19). The T-cell response was detected *in vitro* by a proliferation assay and *in vivo* as p97-specific DTH. Immunization with soluble p97 was found also to induce antibodies but no DTH (19). In this paper, we have evaluated the ability of the immunized mice to reject mouse tumor cells expressing p97. We used both the E line and the B line, which express similar levels of p97 according to binding assays on bulk populations of cells with an antigen-capture assay (17, 27) but which differ with respect to the intercellular variability in p97 expression, as shown below.

Immunity to transplants of p97-positive syngeneic mouse melanoma cells was assessed 2 weeks after either a primary or a secondary immunization with v-p97NY; immunization with parental vaccinia virus (v-NY) was a negative control. In the first experiment (Fig. 1), immunized mice were challenged with the E line, which was injected intravenously at a dose of 5×10^5 cells per mouse and produced lung metastases. Fewer metastases were seen in the groups immunized with v-p97NY than in the controls ($P < 0.005$), while there was no protection against transplants of cells from the p97-negative par line (data not shown). Immunization with purified p97 had no antitumor activity despite its ability to induce anti-p97 antibodies (19). This argues for the use of v-p97NY, rather than p97 protein, as an immunotherapeutic vaccine. It is noteworthy that mice immunized with soluble p97 do not form DTH, since there is evidence that DTH plays a major role as a host defense mechanism against antigenic tumor cells (28).

A second experiment was done to examine the effect of vaccination with v-p97NY on the survival of mice challenged intravenously with a lower dose of cells from the E line (1×10^5). Mice vaccinated three times with v-p97NY showed prolonged ($P < 0.005$) mean survival time (46 ± 15 days) as compared to control (26 ± 3 days), and survival time was prolonged also in mice vaccinated twice with v-p97NY (Fig. 2). All of the mice vaccinated twice and 83% of the mice

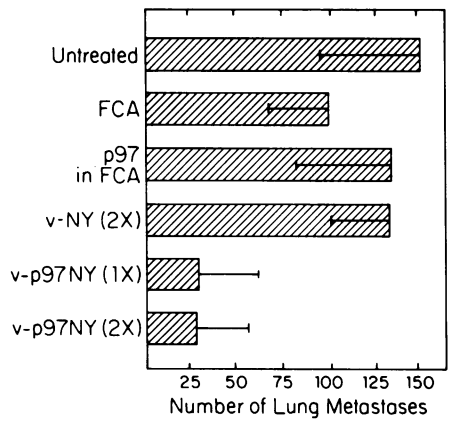

FIG. 1. Effect of vaccination on the formation of lung metastases in mice injected intravenously with E-line cells. Mice (five per group) received either one or two inoculations (1 week apart) of 2×10^6 pfu of v-p97NY or v-NY or of 100 μg of purified p97 in Freund's complete adjuvant, followed 1 month later by a 50-μg booster of p97 in saline. Two weeks after the last vaccination, 5×10^5 E-line tumor cells were injected in the tail vein. Another 2 weeks later, the lungs of the mice were stained by intratracheal injection of 15% India ink, and metastases were counted.

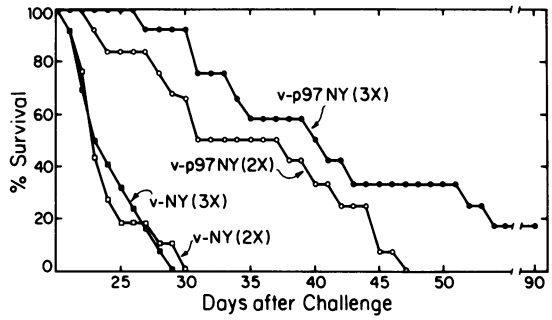

FIG. 2. Survival of prevaccinated mice after intravenous challenge with E-line tumor cells. Mice (12 per group) were immunized either two or three times over a 2-month period with v-p97NY or v-NY (2×10^6 pfu) by tail scarification. Two weeks after the last immunization, they were challenged intravenously with 1×10^5 E-line cells, and death with tumor was recorded.

vaccinated three times eventually died with tumor.

Fig. 3 depicts an experiment similar to that in Fig. 1, but where, instead, cells from the B line were injected intravenously into immunized mice. Very few lung metastases were observed ($P < 0.005$), although a 5-times-greater cell dose (5×10^5 cells per mouse) was used than with the E line.

v-p97NY Therapy of Existing Micrometastases. To determine whether immunization with v-p97NY could affect tumor cells already growing in mice, vaccination was performed 2 days after intravenous inoculation of E cells (Fig. 4). A challenge dose of 5×10^4 cells per mouse was chosen to facilitate the effectiveness of the therapeutic vaccination. The v-p97NY-immunized mice lived longer than v-NY-immunized mice (49 ± 16 days as compared with 35 ± 6 days, $P < 0.005$), and 20% of them survived for 3 months, at which time they were killed and found to be tumor-free.

Even more striking results were seen in an experiment when cells from the B line were used (10^6 cells per mouse) and were injected subcutaneously 2 days before vaccination (Fig. 5). Nine of 10 v-p97NY-immunized mice then remained tumor-free ($P < 0.005$), and in a v-p97NY-immunized group where the mice had been given cyclophosphamide before tumor challenge, all 10 mice survived tumor-free ($P < 0.005$). The reason for giving cyclophosphamide was to diminish immune suppression (29), but the strong effect of immunization with v-p97NY prevented any conclusions regarding the effect of cyclophosphamide.

FACS Analysis of Cell Lines Used for Tumor Challenge. Our data demonstrated a much more striking vaccination effect against the B line than against the E line (compare, e.g., Figs. 1 and 3). To study p97 expression by these two lines at the cellular level, FACS analysis was performed. It

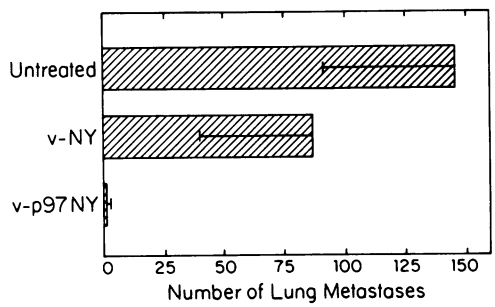

FIG. 3. Prevention of outgrowth of B-line tumors in the lung. Mice (five per group) received three immunizations over a 2-month period with v-p97NY or v-NY (2×10^6 pfu) by tail scarification. Two weeks after the last vaccination, they were challenged intravenously with 5×10^5 B-line cells. Two weeks later, the lungs were stained with India ink, and metastases were enumerated.

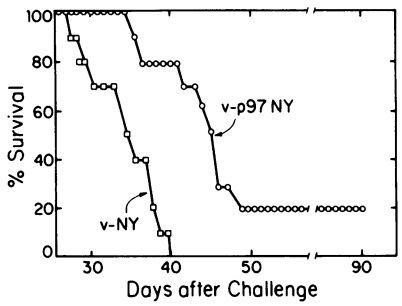

FIG. 4. Survival of mice vaccinated after injection with E-line tumor cells. Mice (10 per group) were injected intravenously with 5×10^4 E-line cells. Two days later, they were vaccinated with v-p97NY or v-NY, followed by two additional vaccinations, which were administered at weekly intervals. Death with tumor was recorded.

is clear from the histograms in Fig. 6 that the E line contains a substantial population of p97-negative cells (estimated at 41%), and this is in contrast to the homogeneously p97-positive cells of the B line. Assessment with both the antigen-capture assay (17, 27) and FACS analysis further demonstrated that the tumors that grew out in v-p97NY-immunized mice challenged with E cells were p97-negative. This is akin to immune selection reported in other systems (30) and is a reflection of the antigenic heterogeneity of tumor cell populations (20, 21).

It is of considerable interest that a few of the vaccinated mice receiving E cells survived tumor-free (in four different experiments, 1/5, 2/10, 0/12, and 2/12 mice, respectively, as compared to 0/39 control mice). We speculate that the immune response destroyed the substantial p97-negative cell population known from the FACS data to be present in the challenge. We further speculate that this "bystander killing" may be caused by an activation of macrophages at the local tumor site, since immunization with v-p97NY induces DTH, and tumor cells are highly sensitive to killing by activated macrophages (28, 31).

Since killing of "bystander cells" could have clinical relevance in view of the heterogeneity of tumor cell populations, preliminary experiments were performed to obtain more direct evidence for the killing of such cells. Fig. 7 illustrates one of three such experiments, all of which gave similar data. In these experiments, either mixtures of both p97-negative (par) and p97-positive (A line) cells, or p97-negative par cells alone were injected into separate footpads of mice, and tumor outgrowth was measured afterward. The

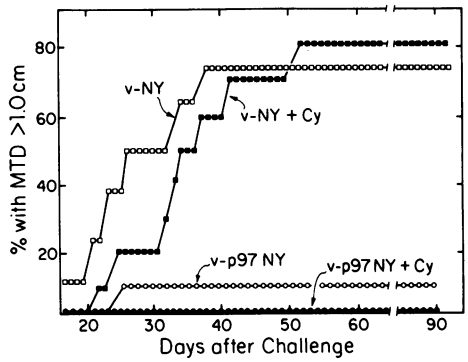

FIG. 5. Tumor growth in mice vaccinated after injection with B-line tumor cells. Mice (10 per group) were injected subcutaneously with 1×10^6 B-line cells. Two days later, they were immunized with v-p97NY or v-NY, followed by two additional weekly immunizations. Cyclophosphamide (Cy) at 100 mg/kg was injected once intraperitoneally 3 days before tumor inoculation in the two groups indicated. MTD, mean tumor diameter.

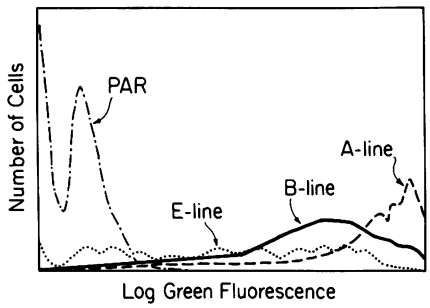

FIG. 6. FACS analysis of p97 expression on mouse tumor cells. Par cells (p97-negative tumor cells) and cells from the p97-transfected A, B, and E lines were first treated with the anti-p97 mouse mAb 96.5 (17), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies by standard techniques (24). Fluorescence profiles of FITC-labeled cells were established by analysis on a FACS IV cell sorter. The abscissa represents a 3-logarithmic range of fluorescence intensity.

A-line cells were chosen because they do not grow in immunocompetent mice, although they are tumorigenic in nude mice (C.D.E., unpublished observations). Addition of the A cells significantly decreased the outgrowth of the par cells ($P < 0.025$ at 1 week, $P < 0.01$ at 2 weeks) (Fig. 7). We interpret these data as follows: an immune response to the p97 antigen expressed on the A-line cells causes local DTH in agreement with previous work (19); this leads to the destruction and/or growth inhibition of the antigen-negative (par) cells at the same site but not at a distant site.

The mouse model used in this paper should lend itself well to studies on how to induce the regression also of large p97-positive tumors, what effector cells are responsible for regression, what roles antigen-specific and nonspecific suppressor cells play, etc. Information obtained from such studies would be helpful when planning therapeutic trials with v-p97NY in humans.

Immunogenicity of v-p97NY in Nonhuman Primates. The experiments presented so far were facilitated by the fact that (human) p97 is foreign to mice, no cross-reactivity between anti-97 mAb and mouse tissue being observed (17, 27). To test whether immunization with v-p97NY can induce an immune response also in a species closely related to man, two adult male *M. fascicularis* monkeys were immunized

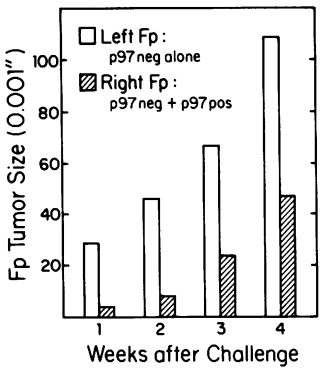

FIG. 7. Reduced outgrowth of p97-negative (par) tumor cells when mixed with p97-positive A-line cells and injected into vaccinated mice (C3H/HeN). Mice (five per group) were immunized once with v-p97NY (2×10^6 pfu). Two weeks later, they were injected in the left footpad (Fp) with 1×10^5 p97-negative (par) cells, which were given in a volume of 20 μl. In the right footpad, a mixture of 1×10^5 par cells and 2×10^5 p97-positive A-line cells was injected in a volume of 20 μl; the A cells do not grow in immunocompetent mice. Average footpad thicknesses were measured in inches (2.54 cm) $\times 10^{-3}$, and the measurements of footpad thickness in control (noninjected) mice were subtracted from these values.

Table 1. Immune response in *M. fascicularis* monkeys immunized with v-p97NY or v-NY

Monkey	Immunogen	Units of p97 antibody	Proliferation index	% killing	
				AbDCC	DTH
1	v-p97NY	7.8	3.9	14.5	–
2	v-p97NY	17.3	9.4	45.6	+
3	v-NY	<0.5	1.1	<1.0	–

Monkeys were immunized and assays were performed as detailed. Sera for the determination of antibody titers and AbDCC were obtained at 4 weeks, blood for proliferation assays was drawn at 5 weeks, and DTH assays were performed at 10 weeks—all after the initial immunization.

three times at monthly intervals with 2×10^8 pfu of v-p97NY by scarification. A control monkey received the same dose of v-NY. These experiments were performed because normal monkey cells express trace levels of a molecule that shows immunological cross-reactivity with human p97 at three of three independent epitopes examined by an antigen-capture assay. Furthermore, mouse anti-p97 antibodies detected human p97 at similar trace levels in normal cells from monkeys and human subjects when used in a binding assay (data not shown). Thus, as far as can be judged, the monkey cells either express human p97 or a very close homologue.

No untoward effects were observed in the vaccinated monkeys over a 3-month follow-up period, and subsequent autopsies gave no indication of tissue damage (W. Morton, personal communication). The two v-p97NY-immunized monkeys made antibodies to p97, and their T cells proliferated when stimulated with soluble p97 in an *in vitro* assay (32) (Table 1). Monkey 2, which showed the stronger responses, displayed DTH with induration and lymphocytic infiltration when challenged intradermally with purified p97. Sera from both v-p97NY-immunized monkeys mediated antibody-dependent cellular cytotoxicity, AbDCC, measured on cells from the p97-positive human melanoma line M-2669.

Although only two monkeys were vaccinated with v-p97NY, it is encouraging that an immune response could be generated, since normal monkey cells express trace levels of p97. This is in agreement with observations made in other systems, where an immune response to tumor-associated "self" antigens has been induced by using the appropriate approach (6). Therefore, the fact that melanoma patients do not appear to have either a cellular immune response or circulating antibodies to p97 (C.D.E., personal observations) does not exclude the fact that p97 can be immunogenic in man, and it has been observed by using an *in vitro* sensitization technique that, indeed, human lymphocytes do have the ability to make anti-p97 antibodies (C. Borrebaeck, personal communication). However, any conclusions about the immunogenicity of v-p97NY in humans will have to await phase I clinical trials. Such trials are also needed to assess whether an immune response to p97, if induced, would have adverse effects on normal human tissues.

We thank Dr. W. Morton of the University of Washington Primate Center with whom the studies in monkeys were performed and Cara Watkins, Stephanie Ashe, and Susan Azemove for skillful technical assistance.

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