vitro for taxol is not significantly different from that of brain tubulin assembly (*10*), and we proposed that the taxol binding sites on these diverse tubulins have been conserved over evolution. Because APM did not inhibit taxol-induced brain tubulin assembly and because taxol sites appear to be conserved, it is unlikely that APM inhibits rose tubulin assembly by competing with taxol for binding to tubulin. Our findings that (i) APM inhibited polymerization at least at the nucleation step, and (ii) maximum inhibition of taxol-induced polymerization occurred at nearly 1:1 molar ratios of APM to tubulin are analogous to the effects of colchicine and podophyllotoxin on taxol-induced brain tubulin polymerization (*19*). These data suggest that APM interferes with rose tubulin polymerization by a mechanism similar to that proposed for the inhibition of brain tubulin assembly by colchicine and podophyllotoxin (*19*). However, the APM binding site appears to be distinct from the low-affinity colchicine binding site on rose tubulin (*8*), since concentrations of colchicine more than 100-fold higher are required for the inhibition of taxol-induced rose tubulin assembly (*9*).

Earlier studies (*20*) have shown that the potent antimicrotubule action of APM can be used to investigate the regulation of tubulin synthesis in the alga *Chlamydomonas*. When *Chlamydomonas* cells are deflagellated and then treated with 2.5 μM APM, flagellar regeneration is completely inhibited (*4*). Weeks and his co-workers (*20*) demonstrated that the induction of tubulin synthesis, which normally accompanies flagellar regeneration, is inhibited by 3 μM APM. Indeed, when used at concentrations below which Ca^{2+} transport may be affected (≤ 5 μM) (*3*), APM appears to be a specific and sensitive probe for such studies. The finding that tubulin synthesis is specifically depressed in animal cells after their treatment with drugs that depolymerize microtubules and presumably increase the tubulin pool size has led to a model of autoregulatory control of tubulin synthesis in eukaryotic cells (*21*). The continued identification and characterization of drugs that specifically depolymerize microtubules in plant cells should facilitate experiments to test whether the proposed autoregulatory model is also applicable to plant systems.

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22. Taxol was provided by M. Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. APM (*O*-methyl-*O*-(4-methyl-6-nitrophenyl)-*N*-isopropyl-phosphorothioamidate) was obtained from Chemagro, Mobay Chemical Corporation, Kansas City, Mo. We thank D. Pepper for performing the indirect immunofluorescence experiments, J. Molé-Bajer for sharing her unpublished results, C. D. Silflow and P. A. Lefebvre for their helpful comments on the manuscript and to G. Erickson for preparation of the typescript. Supported by a grant from Monsanto and by NSF grants 7722398 and PCM 8216035.
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Estrogen Receptor Analysis by Flow Cytometry

Abstract. *A fluorescently labeled estradiol,* N′-*fluoresceino*-N′-(17β-*estradiol hemisuccinamide) thiourea (FE) was used for measuring estrogen receptor content per cell in tumor cells. The cellular content of FE was measured quantitatively by flow cytometry. Binding of FE occurs in the nanomolar concentration range, an indication of the high affinity of the labeled estradiol. Competition of FE for binding sites is observed with estrogens, but not with progestins, androgens, or glucocorticosteroids, indicating the specificity of FE binding. In contrast to other estrogen receptor assays, this new technique requires a small sample size (about 5000 cells) and permits the assessment of heterogeneity in estrogen receptor expression among tumor cells.*

The cytoplasmic receptor for estradiol is present in a subpopulation of patients with breast cancer. The expression of the estrogen receptor in tumors is associated with a longer time interval to recurrence after primary surgery and with a higher degree of responsiveness of metastatic disease to hormonal treatment (*1*, *2*). However, there is considerable variation in the course of the clinical disease among patients with tumors expressing the estrogen receptor. The standard technique for estrogen receptor analysis may provide insufficient information to predict the course of the disease.

Standard technology involves a radioreceptor assay of whole cell extract (cytosol), without regard to normal cell contamination or heterogeneity in estrogen receptor expression among different tumor cells. Cellular heterogeneity can now be investigated in a quantitative fashion with flow cytometry, which permits objective and quantitative analysis

of one or more cellular properties. Flow cytometric analysis is performed with fluorescent probes that bind stoichiometrically to cellular constituents with a high degree of sensitivity and specificity (*3*). Thus, both ploidy and cell cycle distribution can be examined with DNA-specific probes (*4*). In human breast cancer, more than 80 percent of tumors have an abnormal DNA stemline (*5*). We now report on the development and application of a fluorescent probe for quantitative estrogen receptor analysis by flow cytometry.

Earlier attempts were made to synthesize fluorescent probes for studying the estrogen receptor either histochemically or spectroscopically. Thus, fluorescent conjugates of estradiol derivatives covalently bound to protein were examined but failed to fulfill the requirements of specific binding in the nanomolar concentration range (*6*). Alternatively, Danliker *et al.* (*7*) and Barrows *et al.* (*8*) have labeled estrogens directly with fluorescein isothiocyanate; but high concentrations of these probes and their competitors were necessary to demonstrate binding and competition; moreover, the integrity and specificity of these probes have been questioned (*6*). We used *N*-fluoresceino-*N'*-(17β-estradiol hemisuccinamide) thiourea (FE), synthesized by Barrows *et al.*, for the development of an estrogen receptor flow cytometric assay (*9*). The purity of this probe was confirmed by high-performance liquid chromatography and thin-layer chromatography.

Human MCF-7 cells, which contain estrogen receptors, were stained with FE, and the relative fluorescence intensity per cell was measured with a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, California). A 488-nm wavelength was used for excitation by an argon ion laser plasma tube with a constant power output of 500 mW. Two long-pass filters (520 nm) were used to prevent incident light from interfering with emitted light. Light scattering in the forward mode (5° to 18°) was collected simultaneously with fluorescence intensity. Each histogram represents 5000 fluorescence events gated on light scatter to reduce the contribution of fluorescence from cellular debris.

MCF-7 cells stained with 10 n*M* FE showed a fluorescence maximum in channel 133 (Fig. 1A). When cells were incubated with both diethylstilbestrol and FE, fluorescence intensity was reduced by 50 percent. The remaining nonspecific fluorescence exceeded autofluorescence by a factor of 2. Figure 1B illustrates a similar experiment with a breast cancer specimen that contained estrogen receptors (137 fmole per milligram of protein by cytosol assay). In Fig. 1C, fluorescence intensity is plotted as a function of FE concentration, both for total fluorescence and residual (nonspecific) fluorescence after incubation with both FE and estradiol. The subtraction curve indicates the specific FE binding with a saturation level at 6 n*M* FE. A comparison of specific binding curves for a radioreceptor assay and the FE flow cytometric assay showed that the binding of FE to estrogen receptor was about one order of magnitude weaker

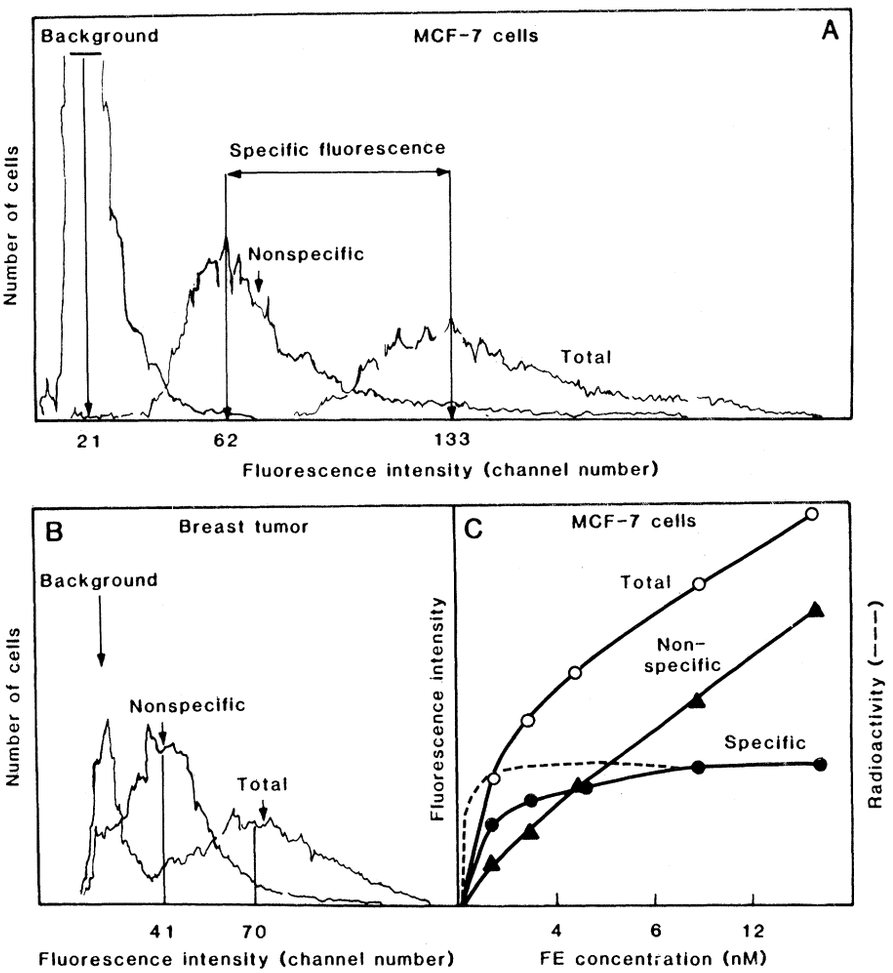

Fig. 1. Flow cytometric assay of estrogen receptor content. (A) MCF-7 cells at a concentration of 10^5 cells per milliliter were grown in minimum essential Eagle's medium supplemented with 10 percent fetal calf serum. Two days before assay, cells were placed in medium supplemented with charcoal-stripped fetal calf serum to minimize the effect of added estrogens. For measurement of estrogen receptor content, cells were exposed to 10 n*M* FE for 2 hours at 37°C, in the absence and presence of 1 μ*M* estradiol as competitor. Cell harvest involved detachment with 0.02 percent EDTA in phosphate-buffered saline (PBS) without trypsin and collagenase. Cells were washed with cold PBS. Single-cell suspensions were prepared by syringing through a 23-gauge needle. Specific estrogen receptor fluorescence was determined from the difference between the two modal channel numbers (shown by arrows) of fluorescence distribution curves obtained in the absence (total fluorescence) and in the presence of estradiol (nonspecific fluorescence). Unstained MCF-7 cells were also measured to determine their autofluorescence intensity. (B) Fluorescence distributions of a human breast tumor sample from a 39-year-old woman. A single-cell suspension of the solid tumor was prepared by mechanical mincing with razor blades, grinding the tissue through a stainless steel mesh, and subsequent syringing. Experimental conditions were identical to those described in (A). FE fluorescence could be suppressed by 41 percent after coincubation with estradiol. (C) Saturation binding experiment with flow cytometry. Modal channel numbers of total and nonspecific fluorescence from histogram distributions illustrated in (A) are displayed as data points of binding curves. The specific binding curve was obtained by subtraction. Estradiol competitor concentration was 1 μ*M* for all concentrations of FE. The specific binding curve obtained with the radioreceptor essay with estradiol is shown by the dashed line. Histograms in (A) and (B) are respresentative histograms obtained directly from the screen display of the data. Repeated runs on the same sample yield superimposable curves. Data in (C) were obtained from two independent experiments. Data points were fitted with a least-squares technique.

than the binding of [^{3}H]estradiol. By Scatchard analysis, we found identical binding site concentrations but different dissociation constants for FE (5 n*M*) and [^{3}H]estradiol (0.6 n*M*). The FE probe fulfilled the requirement of hormone specificity, since there was no competition with FE (10 n*M*) by androgen (dihydrotestosterone), progestin, or glucocorticosteroid (dexamethasone), each of which was present at a concentration of 1 μ*M*. Diethylstilbestrol and estradiol were comparable in competition with FE. The specificity of FE binding to estrogen receptor was further investigated by reverse competition experiments in MCF-7 cells and various cytosols prepared from human breast tumors and human and rabbit uterine tissues. Figure 2A demonstrates that FE effectively competes with [^{3}H]estradiol for estrogen receptor both in whole cells and in cytosols. A concentration of FE ten times higher than that of estradiol was required to achieve a 50 percent competition of radioactivity (*10*). Tissue specificity was demonstrated by the absence of FE binding and estradiol competition when cells such as HeLa, Chinese hamster ovary, and human myeloma (ARH 77), which do not have estrogen receptors, were used. Binding of FE to the receptor proved to be heat-labile; estradiol-suppressible fluorescence was completely abolished after exposure of MCF-7 cells to heat at 45°C for 30 minutes.

One of the objections to the use of fluorescent probes for assaying estrogen receptor has been the possibility of nonspecific binding to cell membranes via electrostatic charges, trapping by endocytosis, or diffusion through damaged cell walls. However, these circumstances are not compatible with the low estrogen competitor concentrations required for suppression of FE fluorescence, which occurred well below a 100-fold excess of estradiol or diethylstilbestrol used in the earlier experiments (Fig. 2B). Likewise, other steroids such as progestin, dihydrotestosterone, and dexamethasone in similar concentrations did not alter the intensity of FE fluorescence.

It might also be argued that the competition apparent in Fig. 2A was not due to FE itself but to estradiol released from FE by cellular esterase activity. This was refuted by the following experiment. A rabbit antibody to estradiol specific for estradiol but not for FE (Miles-Yeda, Revohot, Israel) was incubated with various amounts of FE, 1 n*M* [^{3}H]estradiol, and heat-inactivated breast cytosol as a source of esterase activity. Care was taken to subject the cytosol to mild heat treatment (30 minutes at 45°C), just enough to destroy all estrogen receptor binding activity while leaving esterase activity intact (*11*). No competition with [^{3}H]estradiol was detectable, even in the presence of 100 n*M* FE, whereas only 1 n*M* unlabeled estradiol was required to reach a 50 percent competition (Fig. 2C). Thus, FE hydrolysis into estradiol by added esterase activity was insignificant.

To further optimize the conditions for estrogen receptor analysis by flow cytometry, we investigated the stability of FE fluorescence as a function of time and temperature below 37°C. A lower temperature may be more favorable to the stability of estrogen receptor molecules but requires increased incubation time to reach equilibrium and thus may interfere with nuclear translocation of the receptor-estrogen complex (*12*). At 37°C, equilibrium was reached within 1 hour of incubation, and fluorescence intensity remained stable for more than 2 hours, and up to 4 to 5 hours if samples were subsequently placed on ice.

The exact location of the FE-receptor complex is not known. The demonstration of FE fluorescence enhancement (*13*) in the presence of the DNA stain Hoechst 33342 may prove to be a convenient probe for the assessment of the translocation of the estrogen receptor to the nucleus, which may be more closely related to hormone responsiveness of human breast cancer (*14*).

The value of the flow cytometry assay lies in its potential for studying heterogeneity of estrogen receptor expression. When FE and a DNA-specific fluorochrome are used, two-parameter flow cytometric analysis permits the simultaneous assessment of estrogen receptor expression, cell cycle distribution, and ploidy on a per cell basis. Taken together, these measurements may be more informative than estrogen receptor analysis by the radioreceptor assay in establishing criteria for selecting patients for hormonal therapy or chemotherapy. An additional advantage of the flow cytometric assay is the requirement of a small sample size (about one-tenth of that needed for the radioreceptor assay), which makes estrogen receptor analysis of needle biopsies of solid tissues and bone marrow, as well as pleural effusions, an easy task. Thus, we have al-

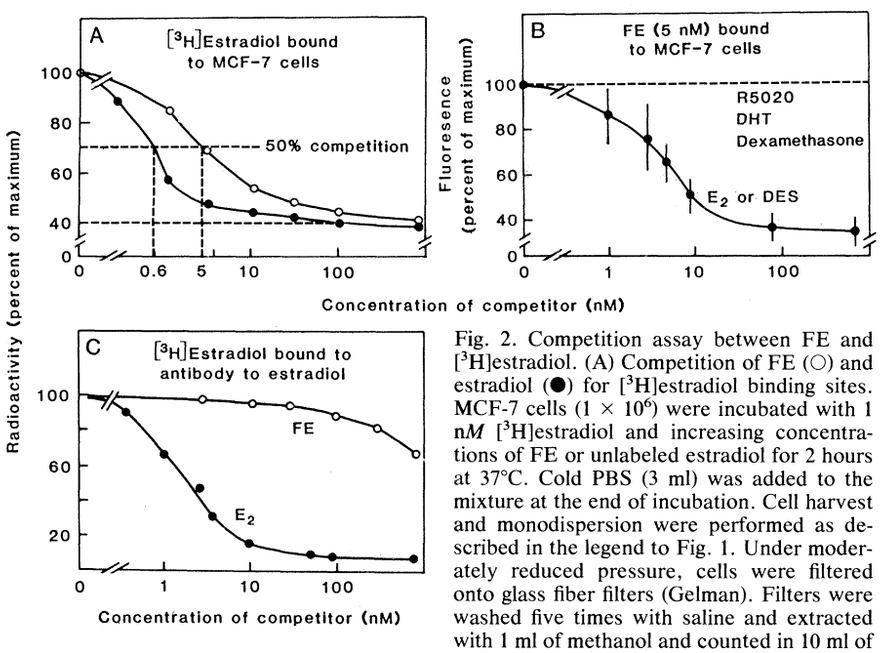

Fig. 2. Competition assay between FE and [^{3}H]estradiol. (A) Competition of FE (○) and estradiol (●) for [^{3}H]estradiol binding sites. MCF-7 cells (1×10^6) were incubated with 1 n*M* [^{3}H]estradiol and increasing concentrations of FE or unlabeled estradiol for 2 hours at 37°C. Cold PBS (3 ml) was added to the mixture at the end of incubation. Cell harvest and monodispersion were performed as described in the legend to Fig. 1. Under moderately reduced pressure, cells were filtered onto glass fiber filters (Gelman). Filters were washed five times with saline and extracted with 1 ml of methanol and counted in 10 ml of Aquasol (Beckman) in a liquid scintillation counter (model 9599, Beckman). Other control experiments (not shown) were done with cytosols (*16*) prepared from various tissues including a primary breast tumor, human uterine tissue (a hysterectomy sample), and rabbit uterine tissue. The apparent dissociation constants K_d for FE and estradiol were 5 to 20 n*M* and 0.8 to 2 n*M*, respectively. Variance was due to different endogenous estrogen levels present in cytosols. (B) Competition in MCF-7 cells of FE with increasing concentrations of estradiol (E_2) diethylstilbestrol (*DES*), or nonestrogenic steroids [progestin (*R5020*), dihydrotestosterone (*DHT*), or dexamethasone]. Decrease in FE fluorescence occurs at competitor concentrations of estradiol and diethylstilbestrol of 1 n*M* and reaches 50 percent at 5 n*M*. (C) Competition of FE and estradiol (E_2) for [^{3}H]estradiol binding sites of rabbit antiserum to estradiol to determine the possibility of FE hydrolysis by cellular esterase. The [^{3}H]estradiol concentration was 1 n*M*, and incubation was conducted for 4 hours at 37°C. Goat antiserum to rabbit serum was added at the end of incubation to precipitate immunoglobulins. Pellets were washed and counted as described above. Data in (A) and (C) were obtained from two independent experiments. Data in (B) were pooled from two experiments.

ready demonstrated that the flow cytometric assay is suitable for the detection of estrogen receptor–positive cells of which there may be less than 5 percent in such samples. Likewise, solid breast tumors with considerable contamination by normal cells and low expression of estrogen receptor as determined by radioreceptor assay (for example, ≤ 5 fmole per milligram of protein) have been successfully identified by flow cytometry (*15*). The reproducibilities of estrogen receptor analysis by flow cytometry and radioreceptor assay compare favorably with a coefficient of variation ranging from 10 to 15 percent.

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Vitamin D_3–Resistant Fibroblasts Have Immunoassayable 1,25-Dihydroxyvitamin D_3 Receptors

Abstract. *Cultured fibroblasts obtained from patients with tissue resistance to 1,25-dihydroxyvitamin D_3 (vitamin D_3–dependent rickets, type II) contain normal, low, or undetectable concentrations of this hormone's receptor protein as measured by a ligand-binding assay. Extracts from these cells were evaluated for receptors by immunoassay with a recently developed monoclonal antibody to the chick receptor. The results show that a protein sedimenting at 3.7S and recognizable by the antibody exists in comparable concentrations in cells from both normal and resistant patients, irrespective of the hormone-binding abnormalities of the cells. This implies that deficiencies in hormone binding associated with inherited tissue resistance to 1,25-dihydroxyvitamin D_3 probably arise from structural variations in the receptor molecule and not from defective receptor synthesis.*

Vitamin D–dependent rickets, type II (VDDR II), is a rare, heritable syndrome clinically characterized by hypocalcemia, secondary hyperparathyroidism, and rickets, all of which persist despite high circulating levels of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (*1–5*). This constellation of features results from peripheral target organ resistance to 1,25$(OH)_2D_3$ comparable to analogous clinical disorders for the glucocorticoid and androgen hormones (*6–9*). Cultured skin fibroblasts from patients with VDDR II have been used to evaluate the underlying defects associated with this malady (*10, 11*). These studies, coupled with accumulating clinical insights, have shown that this disease is heterogeneous and arises almost exclusively from defects in either the receptor's interaction with 1,25$(OH)_2D_3$ or its nuclear site of action (*10–12*).

One particularly abundant phenotype that has been identified in the fibroblasts of a number of different kindreds appears to be a receptor-lacking variant, as deduced by the absence of hormone binding activity. This has led to the possibility that the syndrome is caused by an inherited deletion of the gene for the 1,25$(OH)_2D_3$ receptor. We have now evaluated this possibility by using a radioligand immunoassay (RLIA) to examine these fibroblasts for 1,25$(OH)_2D_3$ receptors (*13*). Our results indicate the presence of normal amounts of material cross-reacting with the receptor antibody in these cells. This suggests that tissue resistance associated with phenotypes in which hormone binding is lacking (HB^-) may be caused by mutations in the binding domain of the receptor protein and is rarely, if ever, the result of genetic deletion.

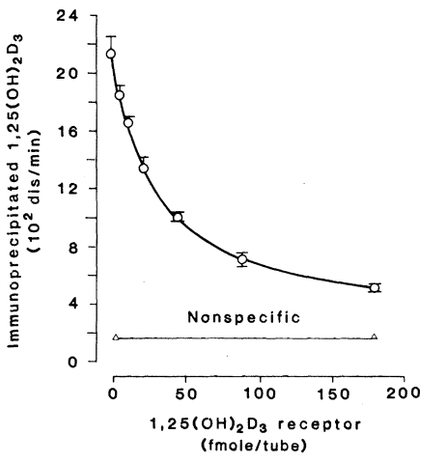

Fig. 1. Standard curve for RLIA. Each point (○) represents the average of triplicate reactions ± the standard error. Reactions were carried out as described. Nonspecific binding (△) was determined in the absence of 9A7γ antibody.

Human fibroblasts were obtained by skin biopsy from normal subjects or from patients, most of whom have been previously described (*1–5, 10, 11*). These cells, as well as the human breast cell carcinoma (MCF-7) and the mouse fibroblast (3T6) lines (American Type Culture Collection, Rockville, Maryland) were maintained in monolayer culture (*8–11, 14*). Cells were harvested at confluence, washed extensively, and disrupted by sonication in KETD buffer (10 m*M* tris-HCl, *p*H 7.4; 1 m*M* EDTA; 0.3*M* KCl, and 5 m*M* dithiothreitol). Cellular extracts used for the 1,25$(OH)_2D_3$–binding assay (*14*) or the immunoassay were obtained after ultracentrifugation.

The 1,25$(OH)_2D_3$ receptors employed in the competition immunoassay were obtained from similarly prepared extracts of chick intestinal mucosa homogenized in KETD buffer. Identical solutions were incubated with either 1,25$(OH)_2[^3H]D_3$ (4 n*M*, 148 Ci/mmole) or 1,25$(OH)_2D_3$ (4 n*M*) to form 1,25$(OH)_2D_3$–receptor complexes. The immunoassay standard curve was created with the 1,25$(OH)_2[^3H]D_3$–receptor complex (80 p*M*), monoclonal antibody (*15–17*), and increasing concentrations of the 1,25$(OH)_2D_3$–receptor complex ranging from 0 to 800 p*M* (*13*) (Fig. 1). Cell or tissue extracts examined by immunoas-