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was observed. Finally, the conjugated drug caused 90% reduction in the size of the lesion 11 days after the initiation of drug treatment. The greatest effect on the regression of the lesions by the conjugated drug was observed at a dose of 1 mg/kg per footpad. The lack of effect at higher concentrations probably reflects saturation of the receptor-mediated uptake process for Mtx-MBSA. The footpad regressed to nearly normal size when Mtx-MBSA was used. In contrast, administration of free Mtx did not significantly affect the footpad lesion. The lesions did not reappear even 4 weeks after the last injection of Mtx-MBSA. During the experimental period all the animals remained healthy with no apparent weight loss. No antibody against MBSA or Mtx-MBSA was detectable in these animals after 3 weeks as determined by the Ouchterlony immunodiffusion technique.

In conclusion, our results show that effective delivery of drug to macrophages can be achieved by using the "scavenger" receptor-mediated endocytic pathway to achieve selective killing of intracellular parasites residing in macrophages, both in vitro and in vivo. A similar approach may be useful for effective delivery of drugs in the treatment of other diseases in which macrophages are the primary target, including tuberculosis, leprosy, monocytic leukemia, and heavy metal storage diseases.

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Studies of the HER-2/*neu* Proto-oncogene in Human Breast and Ovarian Cancer

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Carcinoma of the breast and ovary account for one-third of all cancers occurring in women and together are responsible for approximately one-quarter of cancer-related deaths in females. The HER-2/*neu* proto-oncogene is amplified in 25 to 30 percent of human primary breast cancers and this alteration is associated with disease behavior. In this report, several similarities were found in the biology of HER-2/*neu* in breast and ovarian cancer, including a similar incidence of amplification, a direct correlation between amplification and over-expression, evidence of tumors in which overexpression occurs without amplification, and the association between gene alteration and clinical outcome. A comprehensive study of the gene and its products (RNA and protein) was simultaneously performed on a large number of both tumor types. This analysis identified several potential shortcomings of the various methods used to evaluate HER-2/*neu* in these diseases (Southern, Northern, and Western blots, and immunohistochemistry) and provided information regarding considerations that should be addressed when studying a gene or gene product in human tissue. The data presented further support the concept that the HER-2/*neu* gene may be involved in the pathogenesis of some human cancers.

PROTO-ONCOGENES REPRESENT A family of normal cellular genes that were identified on the basis of their similarity to genetic sequences with known tumorigenic or transforming potential (*1*). Considerable circumstantial evidence now exists that alterations in either the structure, copy number, or expression of one or another of these genes may play a role in the pathogenesis of some human malignancies (*2*). One such gene, called HER-2/*neu* or c-*erb* B2, was first identified by transfection studies in which NIH 3T3 cells were transformed with DNA from chemically induced rat neuroglioblastomas (*3*). The gene encodes a protein that has extracellular, transmembrane, and intracellular domains (*4*) which is consistent with the structure of a growth factor reception.

Recently, we found a 28% incidence of amplification of HER-2/*neu* in 189 primary human breast cancers (*5*). Patients with multiple copies of the gene in DNA from their tumors had a shorter time to relapse as well as a shorter overall survival indicating that gene amplification was prognostic for disease behavior in these individuals. Moreover, multivariate survival analysis showed HER-2/*neu* amplification to be more predictive for clinical outcome than all other known prognosticators with the exception of positive lymph nodes (*5*). Since that initial report, a number of studies have been published on the amplification of this gene

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in human breast cancer and the association of gene amplification with clinical behavior (*6–8*). There is considerable variability in both the reported incidence of amplification and the correlation of gene amplification with patient outcome (*5–10*). Some groups have found amplification rates as low as 10% and no correlation to outcome data while others have found rates as high as 33% and a strong association with outcome (*7, 10*). Given the variable natural history and heterogeneity of human breast cancer, all studies published to date suffer from a similar problem, which is small numbers of evaluated cases (*5–10*). Perhaps a more significant shortcoming of most prior studies of oncogenes in human tumors including our own is that only one aspect of the gene in question (DNA, RNA, or protein status) is evaluated (*5, 11, 12*). The potential errors introduced by dilution of tumor cell macromolecules with macromolecules from surrounding normal vascular, stromal, or inflammatory cells is a general problem in human tumor tissue and a particular problem in breast cancer where these non-malignant cells can account for more than 50% of the tissue. All solid matrix–blotting techniques (Southern, Western, or Northern) are susceptible to these errors. Similarly, these techniques cannot determine whether an observed alteration is specific for only the malignant cells in the tissue.

Studies of the rat *neu* gene isolated from the chemically induced neuroglioblastomas revealed it to contain a single mutation in the transmembrane domain that differentiated it from the nontransforming *neu* gene found in normal rat tissues. This mutation is critical in the conversion of the normal gene into a transforming gene (*13*). To address

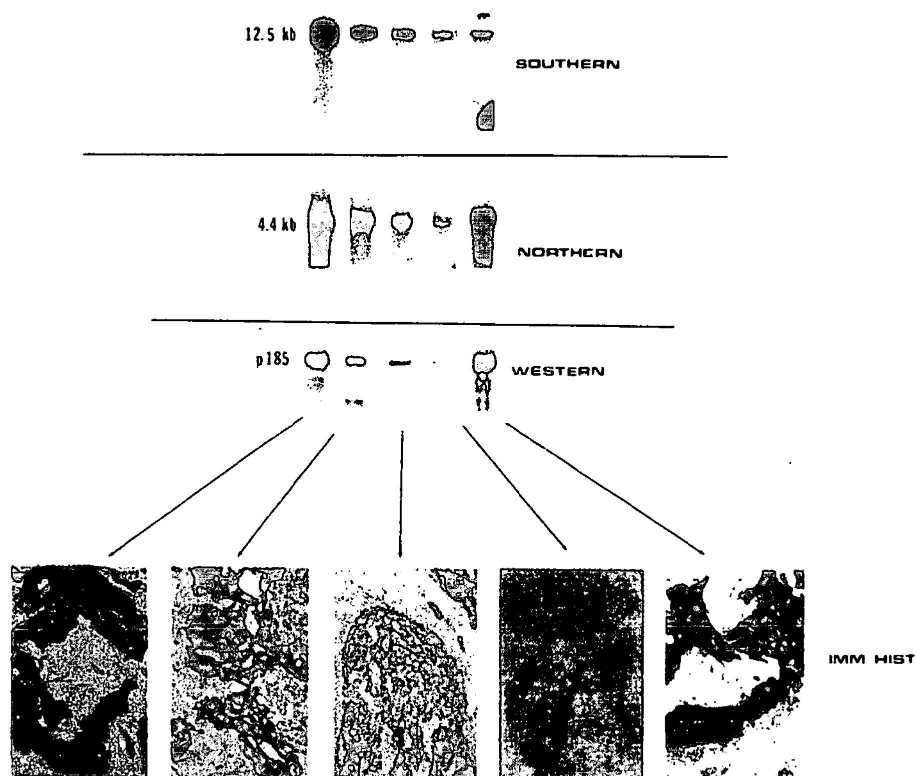

Fig. 1. Examples of the correlation between HER-2/*neu* gene amplification and expression. Southern blot analyses show the 12.5-kb HER-2/*neu* band seen with Eco RI cut DNA. All DNA samples were checked for integrity of high molecular weight species and samples showing evidence of DNA degradation were not evaluated (*9*). Southern blots and HER-2/*neu* copy number determinations were as described (*5, 9*). Hybridizations were done with a 1.4-kb, 3' Eco RI fragment of the human HER-2/*neu* cDNA clone. Northern blot analyses show the 4.5-kb HER-2/*neu* transcript. All RNA samples were checked for integrity of the 28*S* and 18*S* ribosomal RNA species. Total RNA (20 μg) from samples with intact RNA were run on an agarose gel, transferred to a nylon filter, and hybridized with a ^{32}P-labeled HER-2/*neu* probe as described (*11*). Samples with intact DNA but showing some degradation of the RNA were evaluated by slot-blot analysis by loading 12 μg of total RNA on the filter and hybridizing as above. Samples showing degradation of both DNA and RNA were not used for RNA analysis. The relative optical density (O.D.) of bands was determined by soft laser densitometry scanning and ranged from a low of 0.1 O.D. units to a high of 3.8 O.D. units. Tumors were grouped into RNA expression categories as follows: 0.1 to 0.5 O.D. units, 1+; >0.5 to 1.0 O.D. units, 2+; >1.0 to 1.5 O.D. units, 3+; and >1.5 O.D. units, 4+. Western blot analyses show the 185-kD HER-2/*neu* protein band. The relative O.D. of bands ranged from a low of 0.1 O.D. units to a high 4.5 O.D. units. Tumors were grouped into protein expression categories as follows: 0.1 to 0.5 O.D. units, 1+; >0.5 to 1.0 O.D. units, 2+; >1.0 to 1.5 O.D. units, 3+; >1.5 O.D. units, 4+. Immunohistochemical analysis was done as described (*20*) with the anti-HER-2/*neu* specific antibody and frozen sections (*14*). Tissues were scored and placed in one of the four staining categories shown on the basis of the relative level of specific staining as judged by microscopic examination as follows: negative to weak, 1+, 2+, 3+. The five samples analyzed here are arranged in identical order from left to right in each panel. The HER-2/*neu* copy numbers (from left to right) were >20, 5 to 20, 2 to 5, 1, and 1, respectively. The corresponding O.D. readings from the Northern blots were 4.3, 1.4, 0.9, 0.2, and 2.8, respectively. The corresponding O.D. readings from the Western blots were 2.0, 1.1, 0.6, 0.12, and 2.1, respectively. The corresponding immunohistochemistry readings were 3+, 2+, 1+, weak, and 3+, respectively.

Fig. 2. Comparison of immunoperoxidase staining with Western blot on stroma-rich breast cancer. The inset (upper left) is a hematoxylin-eosin stain of a breast tumor rich in stromal tissue. Note the absence of significant numbers of tumor cells. Large middle panel is the immunoperoxidase staining of tumor cells (TC) found in this tissue. The staining is 3+, placing this tumor in the highest category of HER-2/*neu* expression as judged by immunostaining. Southern analysis revealed two to five copies of the gene in the DNA and Northern blot analysis gave an O.D. reading of 0.6 (2+). Western blot of protein from this sample is shown in lane A of the lower right inset. The O.D. reading for this sample was 0.18 (1+), while the Western blot of protein from another specimen with amplified HER-2/*neu* and greater numbers of tumor cells is shown in lane B. The O.D. reading for this tumor (lane B) was 3.2 (4+). Eight of the 11 tumors found to have inappropriately low Western blot data in comparison to other data were similar in that they were stromal-rich tumors.

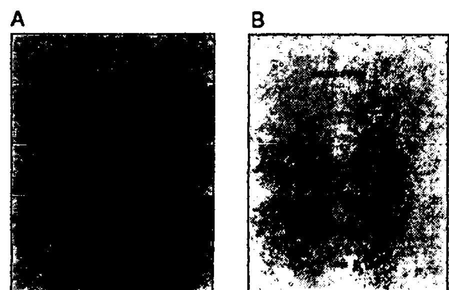

Fig. 3. Comparison of immunohistochemical staining of HER-2/*neu* protein in the same breast cancer specimen evaluated with frozen tissue and formalin-fixed, paraffin-embedded tissue. Tissue shown in panels A and B are from the same tumor which was found to have 5 to 20 copies of the gene and a 2+ expression level by Northern and Western blot analyses. Panel A is the frozen section and shows 2+ immunostaining. Panel B is the formalin-fixed, paraffin-embedded section of the same tumor and shows negative immunostaining.

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the question of whether or not a similar change had occurred in an amplified HER-2/*neu* gene found in human breast cancer, cDNA clones from tumor tissue rather than cell lines were generated by means of the Okayama-Berg vector (*14*, *15*). Tissue was used to circumvent acquired genetic changes which can occur in vitro. Analysis of the transmembrane domain of eight clones from two separate tumors showed the identical sequence. There was no glutamic acid for valine substitution as reported in the transforming *neu* gene from the chemically induced rat tumors (*13*). There was, however, a neutral change of isoleucine for valine at position 655 in the transmembrane domain, which is similar to the sequence found in breast cancer cell lines (*16*). Analysis of the entire coding sequence of full-length clone and comparison with the published placental sequence (*3*) showed no other significant changes (*14*). These data are consistent with the concept that overexpression of a normal HER-2/*neu* gene product rather than mutation to an abnormal gene may be an important pathogenic event for some tumors.

Having obtained and sequenced a full-length cDNA clone from a human breast tumor, we next wanted to generate antisera to the human gene product. The generation and characterization of this antiserum is described elsewhere (*14*) and the antibody is capable of identifying the gene product both by Western blot analysis of tissue homogenates and immunohistochemically in tissue sections.

In the current study, we collected a total of 668 human breast cancer specimens. Of these, 526 had sufficient clinical follow-up to allow for evaluation of an association between gene amplification and disease outcome. As in our initial study (*5*), we performed Southern analysis on samples without knowledge of the clinical outcome. All DNA blots were stripped and reprobed with both p53 and myeloperoxidase probes to evaluate the relative loading of DNA in each lane and to exclude the possibility that "amplification" was caused by partial or complete duplication of chromosome 17 (*9*). Blots were scanned by soft laser densitometry and the level of HER-2/*neu* amplification was determined by the ratio of the HER-2/*neu* signal relative to the single-copy p53 signal.

We evaluated 345 patients with node-positive disease in a blinded fashion (Table 1). Of these, 101 (27%) had evidence of HER-2/*neu* amplification. Univariate survival analysis showed amplification of the HER-2/*neu* gene to be a significant predictor of both disease-free survival and overall survival for these patients (Table 1). Tumor size was slightly better than HER-2/*neu* amplification in the univariate analysis but lost its significance on multivariate analysis, which indicates that it was not independent of nodal status (Table 1). Multivariate analysis showed HER-2/*neu* amplification to be an independent predictor of both disease relapse and overall survival ($P = 0.006$ and $P = 0.045$, respectively) and superior to all other known prognostic factors with the exception of a number of positive lymph nodes for this group of patients (Table 1). We also evaluated DNA from tumors of 181 node-negative patients with a median follow-up of 59 months (62 months for those still alive). Of these, 45 (25%) had amplification of the HER-2/*neu* gene. Univariate and multivariate analysis did not show an association between gene amplification and disease outcome in this group of patients.

Table 1. Univariate and multivariate survival analyses comparing disease-free (relapse) and overall survival to prognostic factors in 345 node-positive breast cancer patients. Statistical analyses were performed by the χ^2 test and by Cox's partially nonparametric regression analysis to evaluate the predictive power of various combinations and interactions of prognostic factors in a multivariate manner as described (*5*). Prognostic parameters evaluated include number of nodes (Nodes), HER-2/*neu* gene amplification (HER-2/*neu*), estrogen receptor (ER), progesterone receptor (PGR), size of primary tumor (Size), and age of patient at diagnosis (Age). The median follow up was 57 months (60 months for those still alive).

	Disease free survival		Overall survival	
	Univariate (*P*)	Multivariate (*P*)*	Univariate (*P*)	Multivariate (*P*)*
Nodes	<0.0001	<0.0001 [0.0818 ± 0.0214]	<0.0001	<0.0001 [0.0912 ± 0.0346]
HER-2/*neu*	0.01	0.006 [0.1142 ± 0.0413]	0.041	0.045 [0.0864 ± 0.0288]
ER	0.235	0.60	0.091	0.157
PGR	0.045	0.07	0.20	0.24
Size	0.003	0.15	0.006	0.16
Age	0.92	0.96	0.20	0.11

*Regression coefficients ± SE are shown in square brackets.

There were 187 tumor samples of sufficient size and integrity to allow for multiple studies in the same specimen. This group of specimens was representative of the overall group in that lesions from both node-negative as well as node-positive patients were included as well as tumors of varying sizes (<1 cm to >2.5 cm). The availability of a cDNA clone for HER-2/*neu* from a human tumor as well as antisera that could identify the protein in both Western blots and tissue sections allowed for a comprehensive evaluation of the gene and its products (RNA and protein) in these tissues. Such a study addresses several critical issues regarding HER-2/*neu* in human breast cancer. First, the correlation between a given level of amplification and relative expression for both RNA and protein is important. Some genes that are amplified in breast cancer, such as *erb* A (*6*), are not expressed; these genes may serve as useful markers but are unlikely to be involved in the pathogenesis of the disease. Second, it should be possible to address the issue of whether amplification and over-expression of HER-2/*neu* is specific to tumor cells in these specimens. When blotting techniques alone are used, there is the risk of dilutional effects, which make it impossible to distinguish signals from tumor cells versus those from normal cells. Third, this approach should give some indication of the relative strengths and weaknesses of the various techniques used in assessing the status of the HER-2/*neu* gene and its products in the same primary human tissue. Studies at both the RNA and protein levels are important since there are examples of human tumors in which transcripts of a particular gene are present but no protein can be detected (*17*). Fourth, if gene expression correlates closely with amplification, a separate assessment of the incidence of amplification can be made.

The samples used for the comprehensive analysis were between 0.5 and 1 g in size and had been stored for various periods of time at −70° to −140°C. Tumor tissue was fractured in liquid nitrogen to obtain a representative piece suitable for cryostatic sectioning and immunohistochemical analysis. The remainder of the entire specimen was ground to powder in liquid nitrogen with a mortar and pestle. This process allowed for an even distribution of tumor cells in all subsequent analyses. A portion of the tissue powder (50 mg) was stored for Western blot analysis and the remainder was extracted for DNA and RNA simultaneously. As in our clinical correlation studies, each procedure was conducted separately and without knowledge of the results obtained by other modalities of evaluation. Southern blots with appropriate p53 and myeloperoxidase controls were performed and HER-2/*neu* copy number determined as described (Fig. 1). Northern blots were performed on 20 μg of total RNA extracted from the sample and were analyzed for the presence and relative intensity of the 4.5-kb HER-2/*neu* messenger RNA using soft laser densi-

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tometry and grouped into one of four RNA expression categories depending on the densitometry results (Fig. 1). Western blots were performed on 100 μg of total protein extracted from each sample and were analyzed for the presence and relative intensity of the 185-kD HER-2/*neu* gene product by soft laser densitometry and grouped into one of four protein expression categories (Fig. 1). Immunohistochemical analysis was performed by an immunoperoxidase staining technique on frozen sections of the tumor tissue. The immunohistochemical specificity of the antibody was previously shown (*14*).

After completion of testing of all samples by each modality, the study was unblinded and a direct correlation analysis between gene amplification and expression was performed. Of the 187 samples evaluated in this manner there was almost complete concordance of the data, with a few notable exceptions. Fifty-one samples (27%) of the cases were called amplified by the Southern blot analyses, and in every instance the amplified samples were shown to over-express the HER-2/*neu* gene product relative to nonamplified cases. In 46 of the amplification-positive samples (90%) there was complete concordance of all tests, that is, amplification correlated with overexpression as determined by Northern, Western and immunohistochemistry. In all 51 cases with amplification, two of the three measures of expression were concordant in showing overexpression.

The Western blot analysis was most discordant, being inconsistent with data obtained by Southern blot, Northern blot, and immunohistochemistry in 11 of the 187 cases (6%). A possible explanation for this phenomenon is that samples showing a weak signal by Western blot may do so because of a relatively large amount of stroma in the tissue. In 8 of the 11 cases in which the Western was discordant, histologic examination confirmed the presence of excessive stromal elements (Fig. 2). DNA and RNA analyses were less sensitive to this problem since there was evidence of amplification and increased transcript levels in these tumors. The increased susceptibility of the Western blot to dilutional effects is likely due to the fact that large amounts of noncellular connective tissue can substantially contribute to the total protein in a sample by adding significant amounts of extracellular matrix proteins such as collagen to the lysate. The stroma, however, is relatively poor in cellularity and will make only minimal contributions to the total DNA or RNA extracted from the same specimen.

The Northern blot analysis was discordant in only four cases (2%) judged amplified at the DNA level, and the immunohistochemical data were inconsistent in only two cases (1%). In each of these cases, the other two modalities used showed overexpression. The correlation between amplification and overexpression ($P < 0.0001$) shown in the comprehensive analysis confirms previously published amplification rates of 25 to 30% for HER-2/*neu* in human breast cancer. There were 18 cases (10%) which were judged as single copy by DNA analysis but which showed clear over-expression at the RNA and protein levels (Fig. 1, lane 5). These cases may represent examples of alterations that occur in control mechanisms for gene expression, rather than increases in gene copy number. Alternatively, these cases may represent instances of true gene amplification at low levels (two- to fivefold) which are missed because of dilution of the tumor cell DNA with DNA from nonmalignant tissue.

Some studies have used only immunostaining of formalin-fixed, paraffin-embedded tissue to assess the status of the HER-2/*neu* product (*8, 10, 18*). This approach presents some problems, since fixation and embedding of tissue frequently decreases or totally destroys reactivity for many antigens (*19*). Furthermore, the degree of loss can vary considerably depending on the duration of fixation with the same fixative (*19, 20*). For 42 of the 187 cases in the comprehensive analysis, formalin-fixed, paraffin-embedded tissue was available. This allowed for a comparison of immunohistochemical staining of the HER-2/*neu* protein in the same specimen prepared in different ways. Evaluation of HER-2/*neu* immuno-reactivity showed that in virtually every case there was some decrease in immunohistochemical staining with the rabbit polyclonal antiserum when comparing fixed to frozen tissue. In tumors expressing very high levels (3+ to 4+), the protein was visible by immunostaining in tissue prepared by either method. The problem was more significant in samples expressing moderate levels of protein (2+) since many completely lost their immunohistochemical reactivity when fixed in formalin and embedded in paraffin (Fig. 3). The majority of these tumors were amplified for the HER-2/*neu* gene at levels of two- to fivefold. However, occasional tumors with 5- to 20-fold amplification that expressed moderate to high levels of protein appeared negative in fixed tissue.

To determine if the same or similar alterations in the HER-2/*neu* gene found in breast cancer occurred in other human malignancies, we screened DNA from 60 non–small cell lung cancers, 40 colon carcinomas, 35 neuroblastomas, and 18 ovarian cancers. There was no significant incidence of HER-2/*neu* gene amplification or rearrangement in any of these groups except for ovarian cancer, where gene amplification was found in 6 of the 18 tumors analyzed. There are several similarities between carcinomas of the ovary and breast. Like breast cancer, the vast majority (>90%) of ovarian malignancies are of epithelial rather than stromal origin (*21*). Also, like breast cancer, steroid hormone receptors are found in the tumor cells of many ovarian malignancies (*22*). Finally, epidemiologic studies suggest that some carcinomas of the breast and ovary may share common etiologic factors since women with breast cancer have twice the expected incidence of ovarian cancer and women with ovarian cancer have a three- to fourfold increased risk of developing carcinoma of the breast (*21*). These similarities and the findings of HER-2/*neu* amplification in the initial cases examined prompted us to evaluate a larger number of ovarian cancers for incidence of gene amplification,

Table 2. Median survival time of patients with ovarian cancer as well as various degrees of HER-2/*neu* gene amplification and expression. All samples were analyzed by both the Kaplan-Meier method and the Cox proportional hazards regression model to provide an assessment of the association of the various assays with survival. The median follow-up was 75 months for those patients still alive. Subgroup categories were as described (Fig. 1).

Assay	Subgroup	Number of cases	Median survival time (days)	Log rank test (P)	Cox proportional hazards regression model (P)*
DNA	1 copy	64	1879		
	2 to 5	17	959	<0.0001	<0.0001 [0.1479 ± 0.0320]
	>5	6	243		
RNA	1+	44	1960		
	2+	16	919	0.0710	0.0244 [0.4537 ± 0.1969]
	3+ to 4+	7	531		
Protein	1+	27	1960	0.1866	0.0628 [0.5437 ± 0.2944]
	2+ to 4+	10	959		
Histochem.	Neg. to 1+	36	1960		
	2+	32	1093	0.0126	<0.0001 [0.4276 ± 0.1163]
	>2+	4	417		

*Regression coefficients ± SE are shown in square brackets.

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