PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186,** 319–326 (1987)

Interferon-γ Induces Altered Oncogene Expression and Terminal Differentiation in A431 Cells[1] (42620)

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Abstract. In tumor cell lines in which oncogene expression is abnormal, modulation of the expression of the oncogene (*myc, src,* or *ras*) by interferons (IFNs) has been observed concurrently with cell growth inhibition or phenotypic reversion. Oncogene expression has also been reported to vary during the differentiation of several neoplastic cell lines. Treatment of monolayer cultures of A431, a human epidermoid carcinoma cell line, with IFN-γ resulted in rapid morphological alterations and cell death not seen with either IFN-α or IFN-β. These changes were accompanied by elevated expression of mRNA's for p21 (the *c-ras* gene product) and the epidermal growth factor receptor as well as increases in the biosynthetic rate of their respective proteins. These effects likewise appeared to be specific for IFN-γ. Growth inhibition by IFN-γ was also observed when A431 cells were grown in a three dimensional *in vitro* culture system. Immunohistochemical staining of these "tumoroids" with a differentiation specific, anti-keratin antibody indicated that IFN-γ enhanced expression of this keratin. This observation suggests that the killing by IFN-γ of A431 cells may result from an acceleraton of terminal differentiation.

Interferons (IFNs) are pleiotropic mediators of cellular activities and have been employed as antitumor agents (10). In certain transformed cell lines overexpressing *myc, src* or *ras* oncogenes, IFN treatment has been shown to reduce selectively expression of the oncogene (1–4). Furthermore, phenotypic reversion or growth inhibition was observed to occur concomitantly with the IFN-induced down modulation of oncogene expression. Specifically, IFN-γ has been observed under a variety of experimental conditions to inhibit growth of both primary human tumor cells and human tumor cell lines (11–14). The mechanism(s) by which IFNs exert their antitumor effects remains obscure.

Oncogene expression has been reported to vary during differentiation of several neoplastic cell lines (5–8).

We have been examining the effects of IFNs on various human tumor cell lines that display abnormal expression of oncogene products. A431 is one such line. These cells maintain large numbers of epidermal growth factor receptor (EGFR) on their surface (approximately 3×10^6 receptor sites/cell (15, 16)) and contain amplified copies (~30-fold) of the EGFR gene (17). In addition to this amplification, evidence has been presented suggesting that EGFR gene rearrangement has also occurred in a portion of the copies contained in A431 cells (18). This rearrangement is associated with the overexpression of an aberrant 2.9-kb mRNA species differing at the 3′ end from the normal EGFR 10- and 5.6-kb mRNA species. Consequently, in addition to the membrane associated EGFR, A431 cells secrete a truncated EGFR representing the extracellular portion of the receptor (19). Although the EGFR gene shares homology with the v-*erb* B oncogene (20), it is not known whether the amplified or the rearranged EGFR genes are involved in the transformed state of A431 cells. Upon treatment of A431 cells with IFN-γ but not IFN-α nor IFN-β, expression of the 10-kb

[1] The opinions or assertions contained herein are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense.

0037-9727/87 $1.50

EGFR mRNA species is dramatically increased. In addition, we found a relationship between treatment of A431 cells with IFN-γ and induction of differentiation, concomitant with the increased EGFR expression, leading to extensive cell death.

Materials and Methods. *Molecular techniques: A. Isolation of cytoplasmic RNA.* Cells are pelleted (1000*g*) after being washed in cold 1 × PBS. The cell pellet is resuspended in lysis buffer (0.5% Triton X-100; 150 m*M* NaCl, 3 m*M* $MgCl_2$, 0.25 *M* sucrose, and 10 m*M* Hepes/NaOH, pH 8.0) by gentle pipetting. Nuclei and cells are removed from the lysate by low speed centrifugation (1000*g*). Supernatant is extracted once with an equal volume of phenol plus 0.5% SDS, 1 m*M* EDTA, 100 mM Tris, pH 9.0, solution (extraction buffer), once with an equal volume of phenol-extraction buffer:chloroform:isoamyl alcohol (25:24:1) and then with chloroform:isoamyl alcohol (24:1) until the interface is clear. RNA is precipitated with 2 vol of absolute ethanol plus 0.2 *M* sodium acetate, pH 5.5, overnight at −20°C. The RNA is spun down at 15,000*g* for 30 min, dissolved in diethyl pyrocarbonate-treated H_2O, and frozen at −70°C.

B. Hybridization. Hybridizations are performed at 40°C in a solution of 50% formamide, 0.1% Denhardts solution, 5× SSC, 0.01 *M* Tris, pH 7.5, 0.25 mg/ml salmon sperm DNA, and 10% dextran sulfate for 16–18 hr. Blots are washed with 2× SSC–0.1% SDS three times at room temperature followed by one or two washes at 50°C in 0.1× SSC–0.1% SDS and then autoradiographed at −70°C (21, 22).

Biosynthetic labeling of cells. Cell lysis, immunoprecipitation of 2×10^7 cpm TCA-precipitable protein, SDS–PAGE, and autoradiography were as described previously (23).

Cell culture and histology. Methodologies for cell culture and processing for microscopy have been described (24).

Results and Discussion. IFN-γ treatment of A431 cells yielded striking results. Cells exhibited an altered morphology prior to extensive cell death within 72 hr of treatment with 200 u/ml of IFN-γ. This effect was seen with both immune IFN-γ and recombinant IFN-γ. Untreated A431 cells grew as compact colonies (Figs. 1A and 1E). With IFN-γ treatment, flattened cells appeared within 24 hr; floaters were not noted at this time (Fig. 1B). By 48 hr floating and pyknotic cells and bare areas of the culture dish were observed (Fig. 1C). At 72 hr approximately 60–70% of the cells appeared rounded and detached from the culture substrate. The remaining, adherent cells were elongated and many had extended processes (Fig. 1D). These effects were even more pronounced at 96 hr (Fig. 1H). At concentrations up to 600 u/ml neither IFN-α nor IFN-β produced similar effects on A431 cells. Some cell flattening by 96 hr of treatment with 200 u of IFN-α or IFN-β was noted (Figs. 1F and 1G).

We have examined the expression of several cellular genes during the course of the IFN-γ treatment described above. Using an EGFR cDNA probe, four transcripts (10, 5.6, 4.8, and 2.9 kb) were detected in total cytoplasmic RNA extracted from treated and untreated A431 cells. These multiple EGFR transcripts have been previously reported in A431 cells (19, 25, 26). After 24 hr of IFN-γ treatment we observed an elevation specifically in the expression of the 10-kb transcript (Fig. 2A). Similar treatment with either IFN-α or IFN-β decreased the level of the mRNA species (data not shown). The effect of IFN-γ on the level of 10-kb EGFR mRNA was time dependent (Fig. 2B). The kinetics of maximal effect (24 to 48 hr) and the magnitude of the effect (up to 10-fold) varied depending upon the IFN-γ preparation but the effect was observed with several preparations of human immune IFN-γ as well as recombinant IFN-γ. The level of c-Ha-*ras*-specific mRNA was also elevated by IFN-γ treatment albeit to a lesser extent than that seen with the EGFR mRNA. The increase and subsequent decrease in Ha-*ras* p21 expression occurs more rapidly than that of the EGFR after IFN-γ treatment. There is a two- to three-fold increase in *ras* mRNA 2 hr post-IFN-γ treatment which decreases to very low levels at times of treatment exceeding 24 hr. These results are consistent with the hypothesis that these genes may be influenced by related control mechanisms due to similarities between the promoter regions of the EGFR and Ha-*ras* p21 genes (27).

In order to determine that the A431 cells were responding to IFN-γ treatment in a characteristic manner, the expression of two genes, 2′5′ oligo A synthetase and HLA class

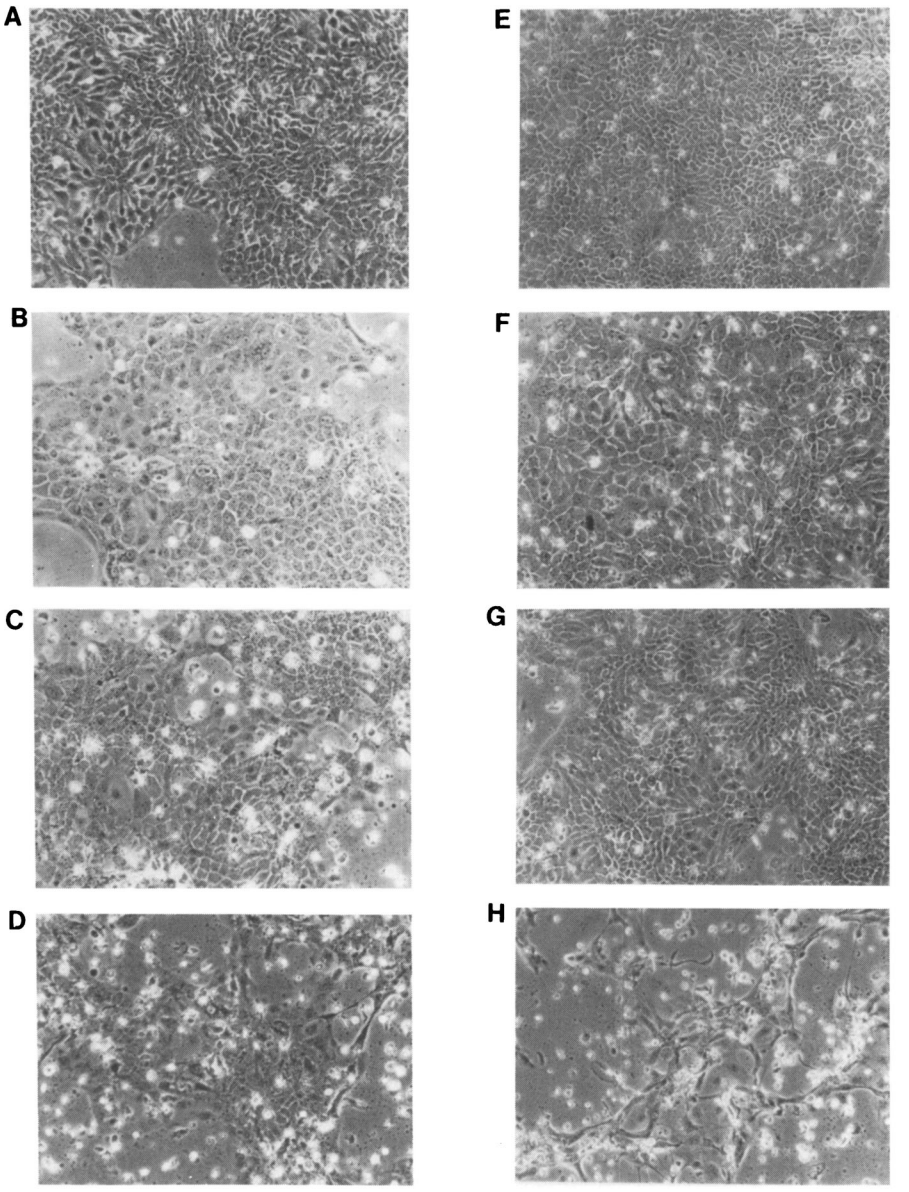

FIG. 1. Effect of IFN treatment on A431 monolayers morphology (A–D). A431 cells were cultured for 72 hr in DMEM plus 10% FBS with (200 u/ml) and withut IFN-γ. IFN addition was made at various times such that cells received no IFN-γ (A), IFN-γ for the last 24 hr of culture (B), for the last 48 hr of culture (C), or for the entire 72 hr (D). (E–H) A similar experimental protocol was followed except that culture was for 96 hr during which the cells received no IFN (E), 200 u/ml IFN-α (F), 200 u/ml IFN-β (G), or 200 u/ml IFN-γ (H). Where treatment times exceeded 48 hr fresh media containing IFN was replenished at 48 hr.

II-DR, previously demonstrated to be induced by IFN-γ (28, 29), was evaluated. The level of mRNA encoding 2′, 5′ oligo A synthetase was slightly elevated in the A431 monolayer by the IFN-γ treatment (Fig. 2B) as expected. The level of transcript for HLA class II DR was also increased (data not shown). The specificity of IFN-γ induction was confirmed using a probe for the "housekeeping" gene glyceraldehyde-3-phosphate

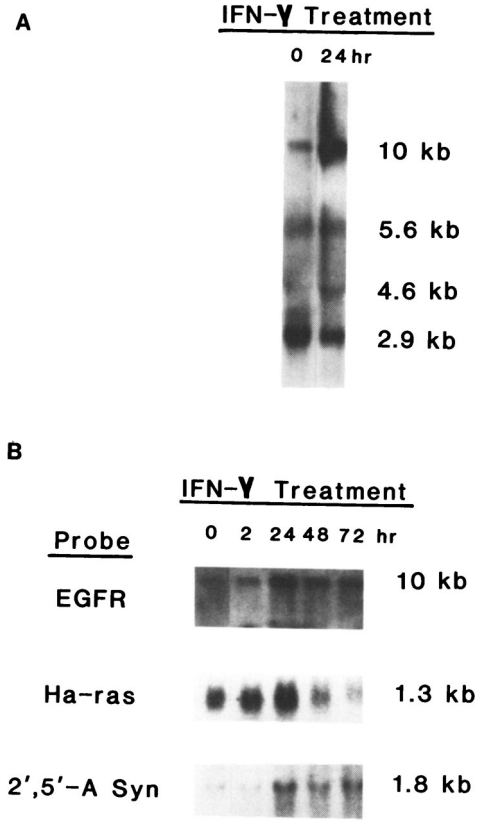

FIG. 2. Effect of IFN-γ on expression of selected genes in A431 cells. A431 cells were treated for the indicated times with 200 u/ml IFN-γ as described in Fig. 1. Cytoplasmic RNA was extracted and 30 μg was subjected to Northern analysis using a nick-translated [^{32}P]EGFR cDNA probe (A). (B) A blot was successively hybridized with the indicated probes and the relevant regions of the resultant autoradiographs are shown.

dehydrogenase (30); this gene is unaffected by the treatment protocol (data not shown).

To establish that the significant increases in the transcript levels of both EGFR and Ha-*ras* were manifested at the protein level, biosynthetic labeling of A431 cells with [^{35}S]methionine was undertaken. Specific immunoprecipitation of the EGFR revealed that the alterations in the level of the 10-kb transcript were reflected in elevated 170-kd EGFR synthesis (Fig. 3A). The biosynthetic rate for the 21-kDa Ha-*ras* product was also elevated reflecting the higher p21 mRNA levels (Fig. 3B). In contrast, biosynthesis of the transferrin receptor was shown to be unaffected by IFN-γ treatment in this time frame. It has been reported that elevation in EGF binding sites per cell accompanies increasing density of A431 cells (31). It would appear unlikely that the increase seen with IFN-γ treatment in our study is a result of increasing cell density as the density is decreased by IFN-γ-induced cell death.

To examine further the biological effects of IFN-γ on A431, cells were grown in a long-term, three-dimensional culture system which we have previously shown permits single tumor cell suspensions inoculated onto agar discs (1×10^6 cells per disc) to form aggregated tumor masses or "tumoroids" with *in vivo*-like histotypic architecture and expression of cellular functions not found in monolayer (9, 24, 32). Cultures were grown up to 29 days with (1200 u/ml) and without IFN-γ; media was changed three times weekly with respective IFN levels mantained throughout the incubation periods. Tumoroids were removed at specific times, fixed in Bouin's solution, paraffin embedded, and serial sections were prepared for histological staining (9). Hematoxylin and eosin-stained sections showed elements reminiscent of *in vivo* squamous cell carcinoma, i.e., keratin pearls and intercellular bridges (Fig. 4B, arrow). Compared to controls, IFN-treated cultures exhibited increased cellular stratification similar to that observed in stratifying squamous epithelia and were consistently reduced in both thickness and diameter (Figs. 4C–4F). Moreover, increased areas of pyknosis were seen in treated cultures (Figs. 4D and 4F).

Since cytokeratins have been shown to be useful markers for epithelial cell differentiation (33), immunohistochemical staining (34) with antikeratin antibodies (Abs) was done to determine if the effects observed were connected with the state of differentiation of the A431 cells. Two monospecific, antikeratin Abs were used: one to a 50-kDa keratin, associated with proliferating epithelia, and the other to a 67-kDa keratin, considered differentiation specific (35, 36). Both treated and untreated tumoroids were found to express the two keratins. However, the control cultures (Fig. 4C) expressed significantly more 50-kDa keratin than those treated with IFN-γ (Fig. 4D), whereas the

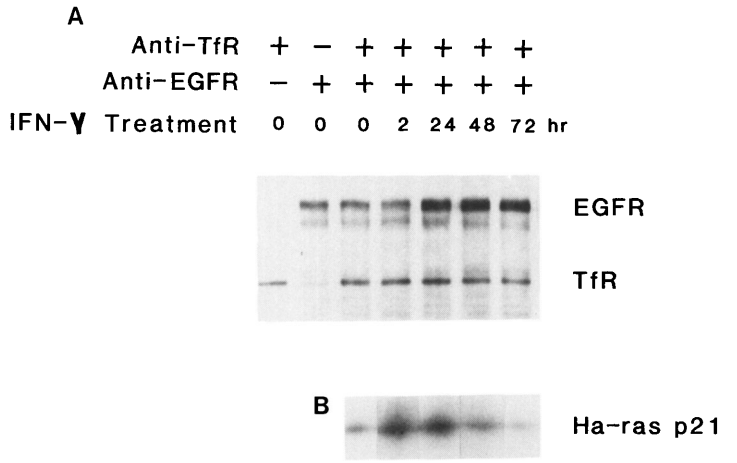

FIG. 3. Effect of IFN-γ on the biosynthesis of EGFR and Ha-*ras* p21 in A431 cells. A431 cells were treated for the indicated times with 200 u/ml human IFN-γ before being incubated with 50 μCi/ml of [^{35}S]methionine in methionine-free growth medium. (A) Anti-EGFR (a gift from Dr. M. Waterfield) and/or antitransferrin receptor (TfR, B3/25, Boehringer-Mannheim) were employed as indicated and samples were analyzed on a 10% acrylamide gel. (B) Anti-p21 (43) (Y13-259, a gift of Dr. M. Furth) was used and a 12% acrylamide gel was employed. The relevant regions of the resultant autoradiographs are shown.

controls (Fig. 4E) expressed less 67-kDa keratin than the IFN-treated cultures (Fig. 4F). These initial results suggest that in this system IFN-γ enhances and accelerates squamous cell differentiation in A431 cells, leading to terminal differentiation and subsequent cell death (34). Further characterization of this biological effect is currently underway.

The question of whether the IFN-γ alteration in expression of EGFR is directly related to the terminal differentiation of A431 cells remains open. However, it has been reported by Jetten that treatment of mouse 3T6 cells with retinoic acids results in an increase in the number of EGFR (37), and a similar increase in EGFR was found to accompany retinoic acid-induced differentiation of mouse teratocarcinoma stem cells (38). Although using different cell lines and other stimuli these studies support the connection between the increased EGFR numbers and the process of differentiation. Two classes of EGFRs exist on A431 cells, with "low-affinity" receptors comprising 90% of the total (39, 40). Boonstra *et al.* (41) have suggested that the ability of epidermal cells to commit to differentiation is inversely proportional to their number of low affinity EGFR. In a related study, King and Sartorelli (39) have isolated variants of A431 that differ in their numbers of EGFR and their sensitivity to growth inhibition by EGF. Variants having lower numbers of EGFR appeared more likely to differentiate in response to serum starvation than were lines with higher numbers of EGFR. The complexity of the process of differentiation may account for what appears to be a contradiction between our studies and those of King and Sartorelli (39); i.e., they argue that lower numbers of EGFR correlate with the tendency to differentiate whereas we observed increased EGFR expression accompanying differentiation.

We have examined a number of other squamous carcinoma cell lines and have confirmed a positive correlation between EGFR expression and susceptibility to killing by IFN-γ (data not shown). In the case of A431 cells, treatment with IFN-γ has been shown to result in higher levels of EGFR mRNA, enhanced EGFR synthesis, and keratin immunohistochemistry consistent with an induction of differentiation. These results are in agreement with those of Nickoloff *et al.* which demonstrated that IFN-γ accelerated differentiation in normal keratinocytes

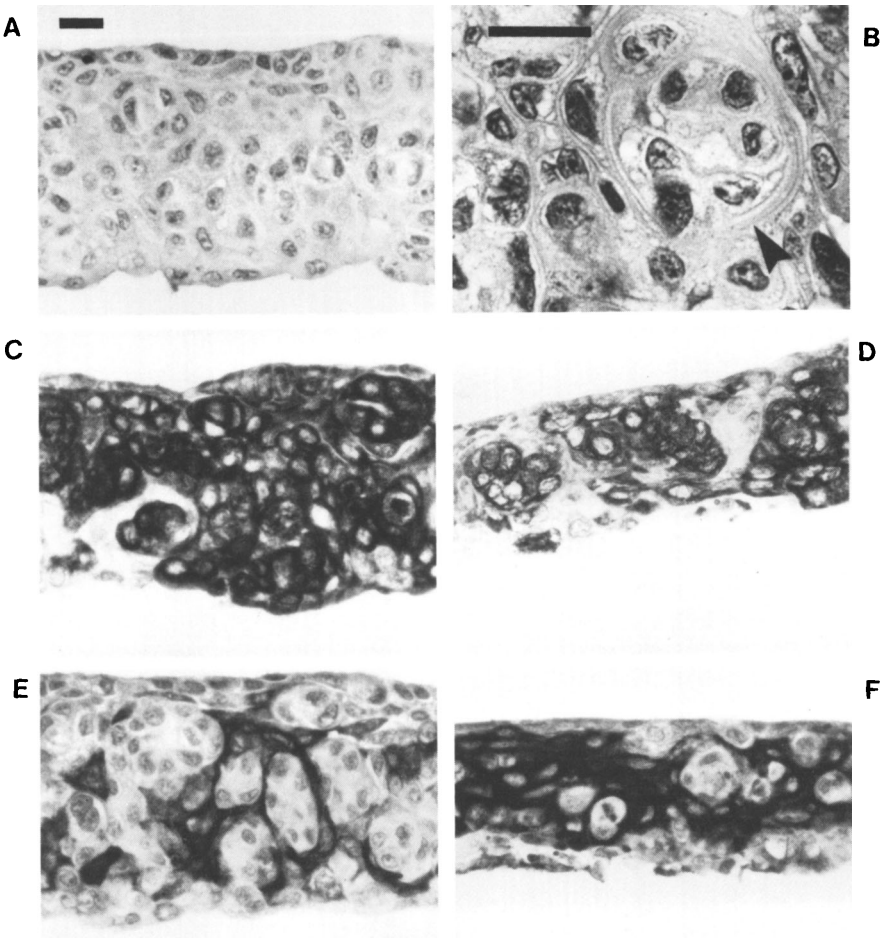

FIG. 4. Features of squamous differentiation in A431 cells grown in three-dimensional culture. A431 cells were grown as three-dimensional agar cultures for 5 days with no IFN (A, B, C, E) or with 1200 u/ml IFN-γ (D, F). Immunohistochemistry was performed with preimmune rabbit serum (A), rabbit anti-50-kDa keratin (C, D), or anti-67-kDa keratin (E, F). (B) Stained with hematoxylin and eosin to reveal keratin pearls and intercellular bridges (arrow). Bars in (A) and (B) represent 20 μm; (C–F) are of the same magnification as (A).

in culture (42). IFN-α and -β have also been shown to promote terminal differentiation and inhibit growth of normal keratinocytes (44, 45). An interferon-like protein in the normal human epidermis has been proposed to serve as a physiological modulator of epidermal growth (46). Terminal differentiation and cell death are in fact the normal course of events in skin cells. The derangement of these normal events results in skin diseases including squamous cell carcinomas. From our findings, it would appear that IFN-γ is capable of inducing the terminal differentiation of a carcinoma cell line.

We thank Drs. D. Roop, M. Waterfield, and M. Furth for monoclonal antibodies against keratins, EGFR, and *ras* p21, respectively; Dr. J. Schlessinger for EGFR cDNA clone; Dr. M. Revel for 2′, 5′ oligo A synthetase cDNA clone; Dr. R. M. Friedman for stimulating discussions; Ms. R. Garner for excellent technical support; and Ms. S. Bowman for manuscript preparation. This

work was supported in part by grants from NIH (CA45158) and USUHS (CO7488) to E.H.C.

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Received May 21, 1987. P.S.E.B.M. 1987, Vol. 186.
Accepted July 30, 1987.