THE DCC PROTEIN AND PROGNOSIS IN COLORECTAL CANCER

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ABSTRACT

Background Allelic loss of chromosome 18q predicts a poor outcome in patients with stage II colorectal cancer. Although the specific gene inactivated by this allelic loss has not been elucidated, the *DCC* (deleted in colorectal cancer) gene is a candidate. We investigated whether the expression of the DCC protein in tumor cells is a prognostic marker in colorectal carcinoma.

Methods The expression of DCC was evaluated immunohistochemically in 132 paraffin-embedded samples from patients with curatively resected stage II or III colorectal carcinomas. The Cox proportional-hazards model was used to adjust for covariates including age, sex, tumor site, degree of tumor differentiation, and use of adjuvant therapy.

Results The expression of DCC was a strong positive predictive factor for survival in both stage II and stage III colorectal carcinomas. In patients with stage II disease whose tumors expressed DCC, the five-year survival rate was 94.3 percent, whereas in patients with DCC-negative tumors, the survival rate was 61.6 percent ($P<0.001$). In patients with stage III disease, the respective survival rates were 59.3 percent and 33.2 percent ($P=0.03$).

Conclusions DCC is a prognostic marker in patients with stage II or stage III colorectal cancer. In stage II colorectal carcinomas, the absence of DCC identifies a subgroup of patients with lesions that behave like stage III cancers. These findings may thus have therapeutic implications in this group of patients. (N Engl J Med 1996;335:1727-32.)

STAGE II or Dukes' stage B2 colorectal cancer accounts for approximately one third of the cases of colorectal cancer diagnosed annually in the United States. Surgery can cure 80 percent of these cases, but the prognosis is poor in the remainder, and unlike stage III colorectal cancer, stage II disease does not benefit from adjuvant therapy.[1-7] A recent study by Jen et al. found that allelic loss of chromosome 18q was linked to the prognosis in patients with stage II colorectal cancer.[8] The retention of both alleles predicted a favorable outcome, whereas the loss of one allele predicted a poor outcome, similar to the outcome of stage III tumors. The determination of chromosome 18q status may thus help stratify patients with stage II disease into good-risk and poor-risk groups.

The specific gene affected by the allelic loss in the colorectal cancers studied by Jen et al. was not identified, but the *DCC* (deleted in colorectal cancer) gene, which is in chromosome 18q21.2 immediately adjacent to the loci evaluated, is a strong candidate. Hahn et al. recently discovered a gene within that region that they mapped to chromosome 18q21.1 and termed *DPC4* (deleted in pancreatic cancer locus 4).[9] This gene, which seems distinct from *DCC*, also has to be taken into account when loss of heterozygosity occurs in chromosome 18q. To further evaluate the *DCC* gene in colon cancer, we examined the expression of the DCC protein in stage II and III colorectal cancers immunohistochemically and assessed its importance as an independent prognostic marker.

METHODS

Patients and Tumor Specimens

One hundred thirty-two formalin-fixed, paraffin-embedded samples from patients with stage II or stage III sporadic colorectal carcinomas were obtained from the archival tumor banks of the Joint Center for Radiation Therapy–New England Deaconess Hospital in Boston, and the Lahey–Hitchcock Medical Center in Burlington, Mass. Curative resections were performed from 1965 through 1975 and 1988 through 1990, respectively. Having been compiled for research purposes, the data from these sources represented groups of patients for whom archival tissue and adequate data on pathological findings and clinical follow-up were readily available. Staging was based on pathological and surgical results. Follow-up for this retrospective analysis was carried out by reviewing the patients' records and contacting the patients' physicians, with results confirmed as of March 10, 1996.

Antibodies

Paraffin-embedded tumor sections were initially evaluated immunohistochemically with a panel of antibodies against DCC. One monoclonal antibody (clone G97-449, Pharmingen, San Diego, Calif.) and three polyclonal antibodies, 721, 723, and 724,[10] all recognizing epitopes in the cytoplasmic domain of DCC, were used. Antibody 721 was raised against a hexahistidine human

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DCC cytoplasmic-domain fusion protein and purified by affinity chromatography on an antigen–agarose column. Antibodies 723 and 724 were raised against a hexahistidine xenopus DCC cytoplasmic-domain fusion protein and purified in a similar manner.[10] The specificity of each antibody was demonstrated by Western blot analysis with tissue from the central nervous system, where DCC is expressed at high levels, and subsequently tested by immunohistochemical staining of colonic tissue. All four antibodies produced an identical pattern of staining of the cytoplasm. Specimens in this study were processed with the arbitrarily chosen antibody 723.

Immunohistochemical Analysis

Individual tissue sections of 4 to 5 μm were deparaffinized and heated in a 10 mM citric acid monophosphate buffer (pH 6.0) for 30 minutes in a 1.35-kW microwave oven (model MW5620T, Samsung, Suweon, Korea) at high power.[11] This method of enhancing the recognition of antigen in archival tissue is termed antigen retrieval. To minimize the evaporation of buffer during heating, the tissue slides were microwaved in a nonmetallic kitchen pressure cooker (Nordicware, Minneapolis). Immunohistochemical staining was performed with either an automated immunohistochemical processor (model 320, Ventana Medical Systems, Tucson, Ariz.) or, manually, with the Vectastain Elite ABC reagent kit (Vector Laboratories, Burlingame, Calif.). The primary antibody was used at a dilution of 1:500. The horseradish peroxidase–conjugated secondary antibodies we used were goat antimouse IgG for the monoclonal antibody and goat antirabbit IgG for the polyclonal serum. Slides were counterstained with methyl green or hematoxylin–copper sulfate bluing reagent, rehydrated, and then mounted with Permaslip solution (Alban Scientific, St. Louis). Controls from each specimen were exposed to phosphate-buffered saline, rabbit preimmune serum, or an isotype-matched irrelevant monoclonal antibody, where appropriate. In antibody-adsorption studies, antibodies were incubated overnight at 4°C in the presence of excess peptide antigen. These preparations were then used in immunohistochemical studies.

The status of DCC was assessed in a coded manner by a surgical pathologist without knowledge of the clinical and pathological features of the case or the clinical outcome. At the outset, samples were to be regarded as positive for DCC when at least 25 percent of the tumor cells were immunoreactive. However, this classification proved to be unnecessary, since staining for DCC turned out to be an "all-or-nothing" phenomenon.

Statistical Analysis

The primary outcome in this study was overall survival, as measured from the date of surgery to the time of the last follow-up visit or death. Data on survival were censored if the patient was still alive at the time of the last follow-up visit or had died from other causes. Survival curves were constructed according to the method of Kaplan and Meier.[12] The sample size was adequate to detect with 90 percent power a hazard ratio of 2 for the risk of death associated with DCC status (positivity vs. negativity) for both stage II and stage III disease. The survival curves for stage II and stage III colorectal cancer were compared on the basis of DCC status with a log-rank analysis. In determining the risk ratio, the Cox proportional-hazards model[13] was used to assess the simultaneous contribution of the following base-line covariates: age (<65 or ≥ 65), sex, site of the tumor (colon vs. rectum), the degree of differentiation of the tumor (poorly differentiated vs. well or moderately well differentiated), the use of radiation or chemotherapy, the tumor–node–metastasis (TNM) stage, and DCC status. All covariates were retained in the model to illustrate the lack of effect in the presence of other significant factors. The distribution of each base-line covariate was compared for DCC-negative and DCC-positive subgroups with the Wilcoxon rank-sum test for continuous data and Fisher's exact test for categorical data. A P value of less than 0.05 was considered to indicate statistical significance. All tests were two-sided.

RESULTS

Immunohistochemical Staining

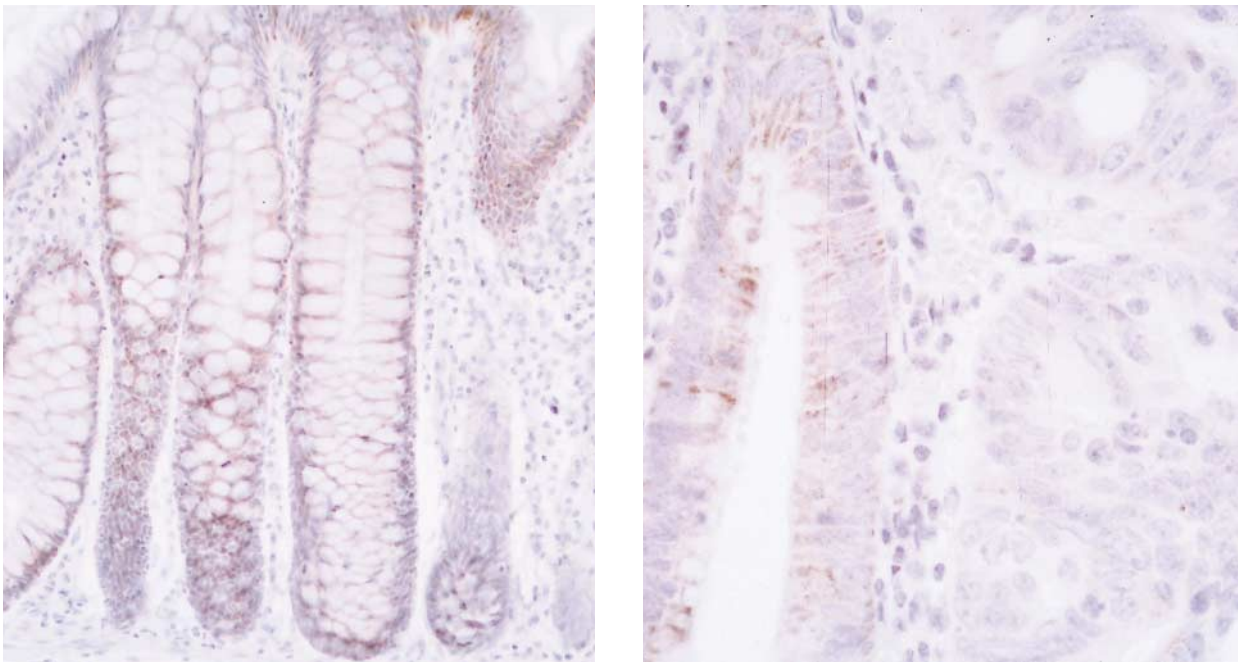
If the antigen-retrieval technique was not used, only faint, patchy staining was observed with the different anti-DCC antibodies. By contrast, after treatment of the sections by microwaving, all four anti-DCC antibodies produced distinct granular cytoplasmic staining in identical patterns (Fig. 1). Staining was abolished when the antibody was first adsorbed with the appropriate peptide antigen (data not shown). Normal colonic mucosa displayed uniform staining of DCC throughout the crypt and luminal epithelial cells; there was no detectable immunoreactivity in nonepithelial cells (Fig. 1A). DCC was also observed in seven of seven incidental adenomatous polyps (Fig. 1B); cells with adenomatous changes and normal mucosa adjacent to the tumor tissue provided positive internal controls for reliably assessing the presence or absence of DCC in the carcinoma. In the cancers in which DCC was detected, a homogeneous pattern of staining was observed throughout the tumor mass (Fig. 1C). Table 1 summarizes the DCC-staining status of the 132 tissue samples.

Characteristics of the Patients

Table 1 gives the relevant clinical characteristics of the 132 patients whose tumors were analyzed immunohistochemically. The study population was evenly divided between men and women, and the mean age was 65.4 years. Neither sex nor age correlated with positivity for DCC ($P = 0.06$ and 0.90, respectively). In approximately two thirds of the patients, the tumor was confined to either the right or left colon; the remaining third had carcinoma of the rectum. There was no difference in the frequency of the absence of DCC in tumors from these sites ($P = 1.00$). Tumors from 50 percent of the patients had no detectable DCC. DCC was absent in 50 percent of the patients with stage II disease and 50 percent of those with stage III cancer. Of the tumors evaluated, 86 percent were either well or moderately well differentiated; 14 percent were poorly differentiated. The TNM stage was not associated with DCC status ($P = 0.31$). Although the majority of patients who received adjuvant therapy were categorized as having stage III cancer, there was no significant difference in this group between those who were DCC-positive and those who were DCC-negative ($P = 0.44$). The mean duration of follow-up was 95.7 months for patients with DCC-positive tumors and 85.1 months for those with DCC-negative tumors ($P = 0.96$).

The Expression of DCC and Prognosis

The overall survival of the patients in our study was consistent with other survival data for colorectal carcinoma.[14] As expected, the TNM stage was an im-



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portant prognostic factor (Fig. 2). The overall 5-year survival rate was 78.0 percent for patients with stage II disease and 46.2 percent for those with stage III disease, with median follow-up times of 74.9 months and 78.5 months, respectively. Figure 3 shows Kaplan–Meier life-table analyses of patients with stage II disease, stratified according to DCC status. The 5-year survival rate for patients with DCC-positive tumors (median follow-up, 74.8 months) was 94.3 percent, whereas the rate was 61.6 percent for patients with DCC-negative tumors (median follow-up, 76.9 months). The 5-year survival rate was 59.3 percent among patients with DCC-positive stage III disease and 33.2 percent among patients with DCC-negative stage III tumors, with median follow-up times of 81.0 and 75.0 months, respectively (Fig. 3). The outcome in patients with DCC-negative stage II tumors was very similar to the outcome in patients with DCC-positive stage III tumors (Fig. 3). At the conclusion of the study, 64 percent of patients with DCC-positive tumors were alive, as compared with 33 percent of patients with DCC-negative tumors ($P<0.001$).

Multivariate Analysis

Multivariate analysis with the Cox proportional-hazards model showed that tumor stage (relative risk of death associated with stage III, 3.1; $P<0.001$) and DCC status (relative risk of death associated with DCC-negativity, 3.2; $P<0.001$) were independent prognostic factors (Table 2), whereas age, sex, tumor site, and adjuvant therapy were not significant independent indicators of prognosis. When

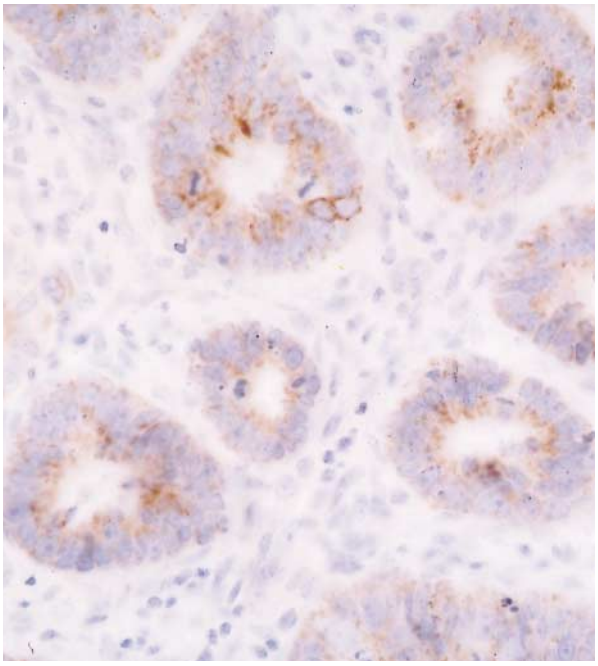

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Figure 1. Immunohistochemical Analysis of the Expression of DCC Protein.

Panel A shows normal colonic mucosa: DCC is expressed uniformly (brown staining) throughout the crypt and luminal cells. In Panel B, the DCC protein stains intensely in the adenomatous tissue on the left, whereas there is no immunoreactivity in the adjacent carcinoma. In Panel C there is homogeneous staining of the DCC protein in a colorectal carcinoma.

TABLE 1. CLINICAL CHARACTERISTICS OF 132 PATIENTS WHOSE COLORECTAL CARCINOMAS WERE EVALUATED FOR DCC.*

CHARACTERISTIC	TOTAL	DCC-POSITIVE	DCC-NEGATIVE	P VALUE
Sex — no. (%)				
Female	66	39 (59)	27 (41)	0.06†
Male	66	27 (41)	39 (59)	
Age — yr	65.4±11.4	65.1±11.9	65.7±10.9	0.90‡
Tumor site — no. (%)				
Rectum	47	23 (35)	24 (36)	1.00†
Colon	85	43 (65)	42 (64)	
TNM stage — no. (%)				
II	70	35 (53)	35 (53)	1.00†
III	62	31 (47)	31 (47)	
Degree of differentiation of tumor — no. (%)				
Good	6	5 (8)	1 (2)	0.31†
Moderate	107	52 (79)	55 (83)	
Poor	19	9 (14)	10 (15)	
Adjuvant therapy — no. (%)				
No	95	45 (68)	50 (76)	0.44†
Yes	37	21 (32)	16 (24)	
Vital status — no. (%)				
Alive	64	42 (64)	22 (33)	<0.001†
Dead	68	24 (36)	44 (67)	
Length of follow-up (mo) — no. (%)	92.1±52.0	95.7±60.0	85.1±31.6	0.96‡

*Plus–minus values are means ±SD.

†The P value was calculated by Fisher's exact test for the comparison of the DCC-positive group with the DCC-negative group.

‡The P value was calculated by Wilcoxon's rank-sum test for the comparison of the DCC-positive group with the DCC-negative group.

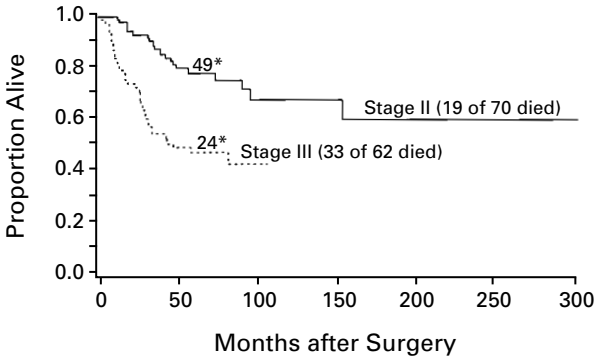

Figure 2. Kaplan–Meier Life-Table Analysis of the Overall Survival of Patients with Colorectal Cancer, According to TNM Stage.

Patients with stage II colorectal cancer had a significantly better outcome than those with stage III disease (P<0.001). The number of patients who died of colon cancer during the entire study is shown in parentheses. The asterisks indicate the number of patients at risk at 60 months.

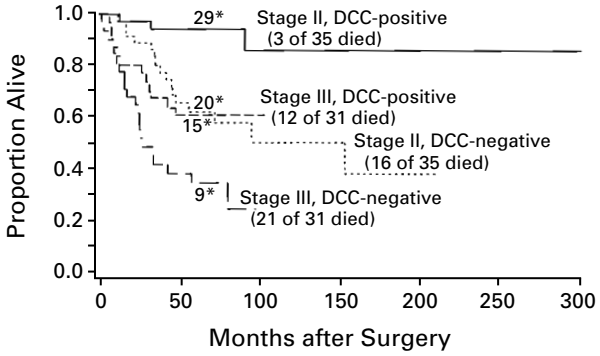

Figure 3. Kaplan–Meier Life-Table Analysis of the Overall Survival of Patients with Colorectal Cancer, According to TNM Stage and the Expression of DCC.

Patients with stage II disease whose tumors were DCC-positive had a significantly better prognosis than patients with stage II disease whose tumors were DCC-negative (P<0.001). Similarly, in stage III disease, patients with DCC-positive tumors had a significantly better overall survival rate than patients with DCC-negative tumors (P=0.03). The number of patients who died of colorectal cancer during the entire study is shown in parentheses. The asterisks indicate the number of patients at risk at 60 months.

the patients were stratified according to stage and temporal cohort, adjuvant therapy was not a significant prognostic indicator (data not shown). An unfavorable tumor grade (poorly differentiated vs. well or moderately well differentiated), by contrast, was predictive of mortality (relative risk, 2.2; P=0.02). The results of the multivariate analysis of maximum-likelihood estimates are given in Table 2.

DISCUSSION

Our results demonstrate that the immunohistochemical assessment of DCC in colorectal carcinomas provides information about prognosis in patients with stage II and III cancers. In patients with stage II disease and DCC-negative tumors, the clinical outcome was similar to that in patients with stage III disease. Patients with DCC-positive stage II tumors, by contrast, had significantly longer overall survival. Half the tumors we studied were DCC-negative, with no significant difference in the frequency of DCC-negative tumors between stage II (50 percent DCC-negative) and stage III (50 percent DCC-negative) cancers. The absence of DCC in stage III tumors was also predictive of a poor outcome, but not to the same extent as in patients with stage II tumors. The only other significant independent prognostic indicators that we found were tumor grade and stage.

Our study of DCC arose from questions about the loss of heterozygosity in chromosome 18q in colorectal tumors and other malignant conditions.[8,15-31] Analysis of the loss of heterozygosity can-

TABLE 2. MULTIVARIATE ANALYSIS OF MAXIMUM-LIKELIHOOD ESTIMATES OF SELECTED CLINICAL VARIABLES IN 132 PATIENTS EVALUATED FOR DCC.

VARIABLE	RISK RATIO FOR MORTALITY (95% CI)*	P VALUE
Age (<65 vs. ≥65)	0.906 (0.503–1.632)	0.74
DCC status (negative vs. positive)	3.155 (1.700–5.852)	<0.001
TNM stage (III vs. II)	3.112 (1.696–5.710)	<0.001
Degree of differentiation of the tumor (poor vs. good or moderately good)	2.218 (1.119–4.397)	0.02
Sex (male vs. female)	1.280 (0.720–2.275)	0.40
Tumor site (colon vs. rectum)	0.868 (0.476–1.583)	0.64
Adjuvant therapy (yes vs. no)	1.209 (0.633–2.309)	0.56

*CI denotes confidence interval.

not pinpoint the lost allele in the deletion region encompassing the *DCC* gene (chromosome 18q21.2), a point highlighted by the mapping of the *DPC4* gene to the same region (chromosome 18q.21.1). Reports of reduced levels of DCC messenger RNA in different kinds of tumors known to have undergone allelic loss of chromosome 18q[25,32,33] support the loss of a *DCC* allele, but immunohistochemical analyses of DCC in tissues, which used several anti-DCC antibodies and frozen tissue sections, gave conflicting results.[34-36] Like others, we observed that frozen sections of normal human colonic tissue did not stain with anti-DCC antibodies. However, by retrieving the antigen with microwaving, we were able to detect DCC in formalin-fixed, paraffin-embedded tissue sections. Under such conditions we found DCC protein throughout the normal colonic mucosa using four different DCC antibodies. Staining in the human cerebellum was confined to the Purkinje cells, verifying previous results with immunostaining and in situ hybridization.[34]

Our immunohistochemical data support the idea that *DCC* is a tumor-suppressor gene. The frequency and types of *DCC* mutations that could impair the function of the DCC protein are unknown. DCC is a transmembrane protein with considerable homology to neural-cell adhesion molecules.[15] Therefore, DCC could participate in the regulation of cell-to-cell or cell-to-substratum interactions and in the control of tumor growth and metastasis. Cultured NIH 3T3 cells expressing the DCC protein stimulate neurite outgrowth in rat PC12 pheochromocytoma cells, suggesting a role for the protein in cell differentiation.[37,38] The disruption of DCC by antisense RNA causes neoplastic transformation of RAT-1 fibroblasts[39] and increases the migratory and invasive properties of a bladder epithelial-cell line.[40] Klingelhutz et al. have restored the expression of DCC in transformed keratinocytes, resulting in the suppression of tumorigenicity, as measured by growth, in nude mice.[41] Recent reports[42-44] demonstrate that DCC possesses netrin-1–binding activity and is probably a mammalian netrin receptor involved in the guidance of developing axons. Although such an association has yet to be established in normal colonic mucosa, it has important implications for the regulation of cell migration and differentiation.

One of the limitations of immunohistochemical analysis is that the detection of a protein by an antibody does not establish its function. The few studies of mutations in the *DCC* gene[21,45] have not shown them to have functional importance. It has yet to be established with known tumor-suppressor genes, or in the case of *DCC,* whether regulatory control of the cell requires a threshold level of the gene product. In our study, the staining results with DCC suggest an all-or-nothing event, and for this reason we did not attempt to quantify the level of DCC protein, as has been done with other tumor markers.[46]

Given the possible role of the *DCC* gene in the pathogenesis of colorectal carcinoma, our finding that DCC status in colorectal cancers provides prognostic information is of particular interest. It seems highly relevant that the absence of DCC in tumors is linked to poor survival among patients with colorectal cancer. Assessment of DCC in colorectal tumors may identify patients with stage II tumors who could benefit from adjuvant therapy. Further understanding of DCC might improve the usefulness of this marker in selecting patients for adjuvant therapy.

Supported by a grant (CA-44704) from the National Institutes of Health (to Dr. Summerhayes).

We are indebted to Dr. David Schoetz, Dr. Anjelica Selim, Mr. William Hamilton, Mr. Ronald Schnirel, and Mr. Jeffrey Martin for their valuable assistance.

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