Monoclonal Antibody KS1/4–Methotrexate Immunoconjugate Studies in Non–Small Cell Lung Carcinoma

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The antigen reactive with murine monoclonal antibody (MAb) KS1/4 is expressed on epithelial malignancies and some normal epithelial tissues. Studies were undertaken to evaluate KS1/4–methotrexate (KS1/4–MTX) immunoconjugate in patients with advanced non–small cell carcinoma of the lung. Eleven patients in two different groups received KS1/4–MTX in two different escalating dose infusion schedules with a maximal tolerated dose of 1,750 mg/M² and a cumulative dose of MTX of 40 mg/M². Toxicities were similar in both groups and included fever, anorexia, nausea, vomiting, diarrhea, abdominal pain, guaiac positive stool, and hypoalbuminemia. Two patients had an associated aseptic meningitis. One patient had a 50% decrease in two lung nodules without a change in lymphangitic infiltrates. This patient received a second course of treatment and developed an immune complex–mediated arthritis and serum sickness. Four patients mounted a human antimouse antibody response. Post-treatment tumor biopsies documented binding of MAb KS1/4. These studies document the feasibility and potential usefulness of a MAb directed against tumor-associated antigens with the targeting of chemotherapeutic drugs in patients with non–small cell lung carcinoma. **Elias DJ, Kline LE, Robbins BA, Johnson HCL Jr, Pekny K, Benz M, Robb JA, Walker LE, Kosty M, Dillman RO. Monoclonal antibody KS1/4–methotrexate immunoconjugate studies in non-small cell lung carcinoma. Am J Respir Crit Care Med 1994;150:1114-22.**

Lung cancer is the leading cause of cancer death in the United States and throughout the world. Of the different histologic types, non–small cell comprises about 80% of lung carcinomas and includes the subtypes of squamous, adenocarcinoma, and large cell carcinoma. Surgical resection has proven to be the best therapeutic option for patients with non–small cell lung carcinoma. However, for the majority of patients, at the time of diagnosis, the disease has spread to regional lymph nodes or to distant sites. Once non–small cell carcinoma spreads beyond the site of origin, the disease is incurable. Aggressive chemotherapy regimens and radiation therapy have met with only limited success.

These investigations are part of ongoing efforts to devise new therapeutic approaches for the treatment of malignancy, particularly for non–small cell lung carcinoma. From a large body of immunologic data, a rationale has been developed during the last decade to suggest that the immune system may have a role in the treatment of cancer. Tumor-associated antigens have been an important part of new approaches in immunotherapy of cancer. These antigens appear as cell surface–associated structures, possibly as a consequence of normal cell surface antigens being altered or expressed by the process of malignant transformation. Monoclonal antibodies (MAb) directed against tumor-associated antigens have the potential to specifically target chemotherapeutic drugs as a therapeutic modality in the treatment of cancer.

The murine monoclonal KS1/4 is an IgG2a antibody that recognizes a carcinoma-associated cell surface antigen which is found on a variety of neoplastic tissues and is expressed by most, if not all, adenocarcinomas (1). The antigen is known to be expressed on a number of normal epithelial cell types, suggesting that it represents an epithelial cell–derived carcinoma marker (2). The sequence of cDNA clones that code for the KS1/4-reactive antigen has been determined (3, 4). These data suggest that the antigen is a 40 kD polypeptide that has heterogeneity in glycosylation, is susceptible to specific proteases and contains a cysteine-rich domain. The antigen that is recognized by MAb KS1/4 may be a suitable target antigen for antibody-directed therapy of non–small cell lung carcinoma (5, 6).

We have previously reported a clinical comparative trial of MAb KS1/4 and KS1/4–methotrexate (KS1/4–MTX) immunoconjugate in patients with non–small cell lung carcinoma (7). Doses and toxicities were defined and compared in both the KS1/4 alone and the KS1/4-MTX patient groups. In general, the infusions were well tolera-

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opsies of carcinoma taken during and after the course of immunotherapy showed selective localization and binding of KS1/4 to the tumor. We found a dose–response relationship between the quantity of administered antibody or immunoconjugate and the subsequent binding of antibody to carcinoma. The KS1/4-reactive antigen is known to be expressed on normal colonic mucosa and post-treatment colonic mucosal biopsies showed binding of MAb KS1/4.

The current studies were undertaken, using higher doses and differing schedules of administration to further evaluate the safety, toxicities, and clinical response of MAb KS1/4–MTX immunoconjugate when administered to patients with non–small cell lung carcinoma. The cross-reactivity of MAb KS1/4 with normal epithelial tissue was further investigated.

METHODS

Preparation of KS1/4–MTX Immunoconjugate

Unconjugated KS1/4 was prepared by Brunswick Biotechnics (San Diego, CA) and was covalently conjugated to methotrexate as previously described (7, 8). As measured spectrophotometrically at 410 nm, the conjugation ratio was 6 mol of methotrexate/mol of antibody (17 mg of methotrexate/g of KS1/4).

Patients

Eleven patients with advanced non–small cell carcinoma of the lung who had received or declined conventional therapies were selected to receive KS1/4–MTX. Eligibility criteria required that each patient's carcinoma express the KS1/4-reactive antigen by immunoperoxidase staining. All patients had measurable or evaluable disease, a performance status of 0–2 on the Eastern Cooperative Oncology Group scale, carcinoma accessible for repeat biopsy, no chemotherapy or radiation therapy for at least 30 d before entry into the trial, and adequately preserved hematologic (hemoglobin > 10 g/dl, white blood cell count [WBC] > 3,000/mm³, platelet count > 100,000/mm³), renal (creatinine < 2.0 mg/dl), and hepatic (bilirubin < 2.5 mg/dl, serum glutamic-oxaloacetic transaminase < 70 units, alkaline phosphatase < 300 units) parameters. Informed consent was obtained based on protocols on file with the Human Subjects Committee of Scripps Clinic and Research Foundation.

Study Plan

The first group of six patients received KS1/4–MTX as a 1-mg test dose, followed by an escalating infusion schedule of 50, 100, 250, 350, 500, and 500 mg/M². The duration of each infusion was 24 h. The longer time of infusion, compared with our initial comparative clinical trial (7), was chosen on the basis of data that suggest toxicities, particularly respiratory symptoms, with high doses of MAb infusions can be lessened or avoided by longer infusion times. The infusions were three times a week for a total of 2 wk. The shorter duration of 2 wk compared with the 3 wk was chosen in an attempt to complete the administration of MAb before the mounting of a maximal human antimouse response. This new protocol administered a maximal dose of 1,750 mg/M² for a cumulative dose of methotrexate of 40 mg/M².

A second group of patients received KS1/4–MTX in a different infusion schedule. Two patients received 350 mg/M² in 24 h for three consecutive 24-h periods, for a total dose of 1,050 mg/M² in 72 h. Two patients received 425 mg/M² for a total dose of 1,275 mg/M² and one patient received 500 mg/M² for a total dose of 1,500 mg/M². This second infusion schedule was designed to possibly avoid some of the observed gastrointestinal (GI) toxicity. We hypothesized that with the initial infusion of KS1/4–MTX, there was binding to GI mucosal epithelium. The previous schedule allowed recovery periods, and it was postulated that the regular, normal cellular turnover of the GI epithelium resulted in newly available GI epithelial receptors available for retargeting with the next infusion, and that this targeting and retargeting might play a role in the escalating and cumulative GI symptoms. This, coupled with the large surface area of the GI tract, may have contributed to toxicity. It was thought that with higher daily doses and a shorter total infusion time, GI epithelium might be saturated early, promote subsequent binding to the carcinoma, and lessen GI side effects. Also, a continuous infusion schedule may favor passive diffusion of macromolecules from the vascular and interstitial space into tumor tissue (9, 10).

After the course of treatment in both groups, biopsies of the tumor were obtained after the last dose of KS1/4–MTX and examined for evidence of *in vivo* binding of antibody. Because of the known presence of the antigen on epithelial derived cell surfaces (1, 2) and the previously reported GI toxicities (6, 7), patients underwent examination of their upper GI tracts pre- and post-treatment with upper gastrointestinal (UGI) endoscopy. Esophagus, stomach, and duodenum were visually examined, photographed and duodenal biopsies were performed pre- and post-treatment. The antrum was biopsied if the epithelial mucosa was visually abnormal. Pretreatment duodenal biopsies were examined for the presence of the KS1/4-reactive antigen. Post-treatment duodenal biopsies were examined for KS1/4 antibody binding and deposition of complement. Endoscopically observed mucosal abnormalities were graded on a scale of 0 through 4. Normal mucosa was graded as 0, change including erythema only was graded as 1, erosions with friability and exudate were graded as 2, frank ulcer craters were graded as 3, and complete mucosal destruction was graded as 4. Serial blood samples were obtained at multiple time points during and following treatment for determination of KS1/4 serum levels and human antimouse levels. Evaluation of the status of the carcinoma were based on radiographic evaluation and physical examination performed at time of entry and 4 wk after initiation of treatment.

Toxicity Monitoring

A clinical research nurse monitored patients closely for any untoward reactions. Grading and monitoring of toxicities were based on a modified toxicity scale (based upon the Biological Response Modifier Program), and approved by the Human Subject Committee of Scripps Clinic and Research Foundation.

Immunohistochemical Staining of Tissue

Detection of KS1/4-reactive antigen was performed using a biotin-avidin immunoperoxidase technique on paraffin-embedded and fresh frozen tissue blocks. Briefly, paraffin sections were deparaffinized, incubated with 0.3% hydrogen peroxide for 15 min followed by 0.25% porcine trypsin for 30 min at room temperature (RT). Fresh frozen cryostat sections were air-dried, and then fixed in acetone at RT for 10 min. All sections were washed in phosphate-buffered saline (PBS), incubated for 30 min at RT with MAb KS1/4 in PBS (1% bovine serum albumin [BSA]), washed again and incubated for 30 min with biotinylated horse antimouse IgG, 3 μg/ml (Vector, Burlingame, CA). The slides were then rinsed and incubated with horseradish peroxidase (HRP) conjugated avidin D 15 μg/ml (Vector). Sections were rinsed and overlaid with 3-amino-9-ethyl carbazole (AEC) peroxidase chromagen (Biomeda, Foster City, CA) for 10 min, rinsed and counterstained in aqueous Mayer's hematoxylin. Positive controls were immunostained with antihuman keratin, AE1/AE3, 1 μg/ml (Boehringer Mannheim, Indianapolis, IN). Negative controls were immunostained with IgG(K)MOPC 21, 1 μg/ml (Organon Teknika, West Chester, PA), a myeloma IgG1, with no known hapten or antigen binding activity.

In vivo binding of KS1/4 antibody to tumor and normal gastrointestinal mucosa was detected by staining for mouse IgG with an indirect immunoperoxidase technique. Cryostat sections were air-dried, washed with PBS, covered for 30 min at RT with biotinylated horse antimouse IgG (Vector) at 3 μg/ml in PBS. Negative controls were stained with biotinylated goat antirabbit IgG, 1 μg/ml (Tago, Burlingame, CA). Sections were rinsed in PBS and covered with HRP-conjugated Avidin D for 30 min. Bound antibody was visualized with AEC chromagen (Biomeda) and counterstained with aqueous hematoxylin.

A biotin-avidin immunoperoxidase procedure was used to detect complement deposition at the antibody–antigen binding site on duodenal and carcinoma specimens. After biopsy, specimens were placed in a citrate buffer (Zeus Scientific, Raritan, NJ) which contains an antiautolytic agent, n-ethyl-maleimide, and a fixative, $(NH_4)_2SO_4$. Specimens were then washed

TABLE 1

SUMMARY OF THE CLINICAL FEATURES, TOTAL DOSES OF KS1/4–METHOTREXATE IMMUNOCONJUGATE, HUMAN ANTIMOUSE RESPONSES, MAXIMAL TOXICITY, AND CLINICAL RESPONSE

Patient Entry No.* and Treatment Schedule	Histologic Cell Type	Age	Previous Treatment	Total Dose (mg)	Dose/M2	Human Antimouse Antibodies	Maximal Toxicity†	Clinical Response‡
02-01	Adenocarcinoma	76	Surgery	608	331	–	2	SD
02-02	Adenocarcinoma	59	None	2510	1755	+	1	PD
02-03	Large cell	53	Chemo	2660	1750	+	1	PD
02-04	Adenocarcinoma	45	Chemo	1330	722	–	3	PD
02-05	Adenocarcinoma	71	None	1520	850	–	2	SD
02-06	Adenocarcinoma	63	XRT, chemo	1250	1250	–	2	SD
03-01	Adenocarcinoma	64	Chemo	1920	1050	+	2	SD
03-02	Adenocarcinoma	75	Surgery	1500	1050	–	2	SD
03-03	Adenocarcinoma	59	Chemo	2490 (1992)§	1270 (1015)§	+	3	MR
03-04	Adenocarcinoma	82	Chemo, XRT	2160	1270	–	2	SD
03-05	Adenocarcinoma	66	Surgery	1332	780	–	3	PD

Definition of abbreviations: chemo = chemotherapy; XRT = radiation therapy; SD = stable disease; PD = progressive disease; MR = minimal response.

* Patients 02-01 through 02-06 received KS1/4– MTX as an escalating dose infusion schedule on alternate days over 2 wk. Patients 03-01 through 03-05 received KS1/4–MTX as a continuous 3-d infusion.

† Degree of toxicity (0 = none, 1 = minimal, 2 = moderate, 3 = severe, 4 = life-threatening).

‡ Clinical response.

§ Retreatment doses.

liquid nitrogen. Fresh frozen cryostat sections of pre- and post-treatment biopsies were air-dried for 1 h, washed in PBS, incubated 30 min with either rabbit anti-C3d (4.7 μg/ml) or rabbit anti-C4c (20 μg/ml) in PBS (1% BSA). Next, the slides were rinsed with PBS, incubated for 30 min with biotinylated goat antirabbit IgG (1 μg/ml) in PBS and rinsed. Sections were incubated another 30 min with HRP conjugated to avidin D (15 μg/ml), rinsed and incubated 10 min with AEC peroxidase chromagen. Negative controls were incubated with normal rabbit serum (1:1,000 in PBS, 1% BSA). Normal human tonsil sections were used as positive controls.

Determination of KS1/4 Serum Levels and Human Antimouse Antibodies

Monoclonal antibody KS1/4 serum levels were measured by enzyme-linked immunosorbent assay (ELISA) as previously described (7). Human antimouse antibodies were measured by ELISA. Aliquots of serum were incubated for 1 h in 96-well microtiter plates which had been coated with MAb KS1/4 (1 μg/well). After washing, plates were incubated for 1 h with HRP-goat antihuman IgA, IgG, IgM 0.1 μg/ml (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Reaction with *O*-phenylene diamine as the chromagen and absorbance reading at 490 was performed. Based upon a standard curve, the absorbance values (μg/ml) human antimouse antibody were calculated.

RESULTS

Clinical Response

The clinical features, total administered doses of KS1/4–MTX immunoconjugate, human antimouse response, maximal toxicity, and clinical response are summarized in Table 1. Clinical response to treatment was evaluated by comparison of measurements of tumor with imaging studies and physical examination made at time of entry and 4 wk after initiation of therapy. Of the 11 patients completed, one patient (03-03) had a minimal response at the first post-treatment evaluation. This patient had a 50% decrease in size of two midlung zone nodules without change in surrounding lymphangitis infiltration. This patient underwent retreatment with KS1/4–MTX. Repeat evaluation showed continued slight decrease in the same areas of nodularity, while other areas were unchanged. Progressive disease was subsequently documented in this patient. Three patients had progressive disease at the first post-

Toxicity

There were no differences in maximal tolerated doses and toxicities between the two treatment infusion schedules (Table 1). Toxicities are summarized in Table 2. Some degree of toxicity occurred in each patient. Fever, anorexia, nausea, vomiting, diarrhea, abdominal pain, guaiac positive stool, hypoalbuminemia, mild anemia (hemoglobin 9.5–10.9), and brief increases in liver transaminases were seen. One patient experienced a mild pancreatitis. Allergic reactions such as pruritus, urticaria, and anaphylaxis were not seen.

One patient experienced an immune complex–mediated arthritis and serum sickness. Patient 03-03 had a clinical response at the first post-treatment evaluation and received a second series of KS1/4–MTX infusions 6 wk after completing the initial infusions. About 36 h into the second infusion, having received a total dose of 2,490 mg previously and the current dose of 1,160 mg,

TABLE 2

SUMMARY OF TOXICITIES

Toxicities	Patients (No.)	Total	% of Total
Fever	4	11	36
Rigor/chills	0	11	0
Anorexia	11	11	100
Nausea/vomiting	10	11	90
Diarrhea	9	11	82
Abdominal pain	10	11	90
Guaiac positive stool	8	11	73
Hypoalbuminemia	7	11	64
Anemia	1	11	9
Transaminasemia	1	11	9
Pancreatitis	1	11	9
Aseptic meningitis	2	11	18
Serum sickness	1	11	9
Anasarca	1	11	9
Urticaria	0	11	0
Pruritus	0	11	0
Dyspnea	0	11	0
Bronchospasm	0	11	0

the patient had fever and an acute, severe, polyarthritis and synovitis involving the small joints of the feet, ankles, and knees. There was no rash. No infectious etiology was identified. Erythrocyte sedimentation rate (ESR) was elevated at 55, creatine phosphokinase (CPK) was normal. Testing for antinuclear antibodies (ANA) was positive at 1:640 in a speckled pattern. Raji cell assay for circulating immune complex was elevated at 225 (reference range < 100 μg aggregated human gamma globulin). Quantitative cryoglobulins were negative. C4d/C4 ratio was 1.5 (normal 0–1.1). These results are consistent with activation of the classic pathway of complement. KS1/4–MTX infusion was held, the patient was treated with corticosteroids and rapidly improved. KS1/4–MTX infusion was reinstituted and the patient completed the scheduled infusion for a total retreatment dose of 1,992 mg. There were no further fever or joint symptoms. He did experience gastrointestinal toxicity. Human antimouse antibody (HAMA) response was positive upon entry for retreatment at 180 μg/ml. The HAMA response at 6 wk after the retreatment was 450 μg/ml.

Two patients had aseptic meningitis. Patient 02-04 developed headache, photophobia, and fever which were temporally related to the immunoconjugate infusions. The cerebrospinal fluid (CSF) was abnormal with a total WBC of 152 (normal 0–5) with 98% lymphocytes and 2% monocytes; protein was elevated to 62 mg/dl with a glucose of 47 mg/dl (concurrent serum glucose was 80 mg/dl). CSF cytology was benign. CSF cultures were negative for bacteria, fungus, acid-fast bacillus (AFB), and viruses. MAb KS1/4 was undetectable in CSF by ELISA. A magnetic resonance imaging (MRI) scan of the head was normal. The patient improved without treatment and returned 1 wk later with recurrent headache but no fever. Repeat analysis of CSF was improved with a total WBC of 40 with 96% lymphocytes and 4% monocytes. Protein had normalized to 24 mg/dl and glucose was 49 mg/dl (concurrent serum glucose was 92 mg/dl). Repeat CSF cytology was again benign and repeat cultures were negative.

Patient 03-05 developed lethargy and fever. The cerebral fluid was abnormal with a total WBC of 176 with 78% polymorphonuclear leukocytes, 2% lymphocytes, and 20% monocytes. Protein was elevated at 79 mg/dl with a glucose of 178 mg/dl (concurrent serum glucose was 250 mg/dl). CSF cytology was benign. CSF cultures were negative for bacteria, fungus, AFB, and viruses. MAb KS1/4 was undetectable in CSF by ELISA. A CT scan of the head was normal. MAb infusion was discontinued, antiemetics were held, and the patient improved over 12 h. Lumbar puncture was repeated several days later and total WBC had declined to 52 with 94% lymphocytes, 5% monocytes, and 1% macrophage. Repeat CSF cytology and all cultures were negative. MAb KS1/4 was, again, undetectable in CSF by ELISA.

Total administered doses, maximal clinical toxicity, and the findings on upper GI endoscopy and the histopathologic abnormalities of the duodenal biopsies are summarized in Table 3. There was no significant difference in maximal tolerated doses or toxicities between the two treatment infusion schedules. Doses at which GI toxicity occurred were as low as 608 mg and as high as 2,660 mg. All patients recovered symptomatically after withdrawal or completion of therapy.

Post-treatment endoscopic findings were variable. The duodenum was abnormal in 6 of 11 patients. Mucosal changes characterized by erosions with friability and exudates were seen in four patients, erythema alone was seen in two patients. The antrum was abnormal in two patients. The characteristic histopathologic abnormality of the post-treatment duodenum was a mild chronic inflammatory change with increases in lymphocytes, plasma cells, and eosinophils within the lamina propria. There was a correlation between the endoscopic and histopathologic abnormalities (Table 3). Two patients (02-01, 02-02) underwent UGI endoscopy with duodenal biopsies 2 wk after completion of therapy and the duodenum had returned to normal, both visually and histopathologically.

In vivo Localization of MAb KS1/4 and Complement Fragments

Pretreatment immunoperoxidase staining of each patient's carcinoma demonstrated expression of the KS1/4-reactive antigen (Figure 1). Post-treatment carcinoma biopsies were examined for evidence of *in vivo* binding of MAb KS1/4. *In vivo* binding was documented at all doses.

Pretreatment immunohistochemical stains of normal duodenal biopsies uniformly demonstrated expression of KS1/4-reactive antigen and post-treatment duodenal biopsies in all patients docu-

TABLE 3

SUMMARY OF TOTAL DOSES, MAXIMAL TOXICITIES, AND UPPER GASTROINTESTINAL ENDOSCOPIC FINDINGS AND HISTOPATHOLOGY OF DUODENAL BIOPSIES

Patient Entry No.* and Treatment Schedule	Total Dose (*mg*)	Maximal Clinical Toxicity†	Endoscopic/Mucosal Abnormalities‡	Histopathologic Abnormalities§
02-01	608	2	2	2
02-02	2510	1	2	1
02-03	2660	1	0	0
02-04	1330	3	2	0
02-05	1520	2	1	1
02-06	2250	2	1	1
03-01	1920	2	0	0
03-02	1500	2	0	2
03-03	2490 (1992)‖	3	2	1
03-04	2160	2	0	0
03-05	1332	3	0	0

* Patients 02-01 through 02-06 received KS1/4–MTX as an escalating dose infusion schedule on alternate days over 2 wk. Patients 03-01 through 03-05 received KS1/4–MTX as a continuous 3-day infusion.

† Degree of toxicity (0 = none, 1 = minimal, 2 = moderate, 3 = severe, 4 = life-threatening).

‡ Post-treatment endoscopically observed duodenal abnormalities (0 = normal, 1 = change including erythema only, 2 = erosions with friability

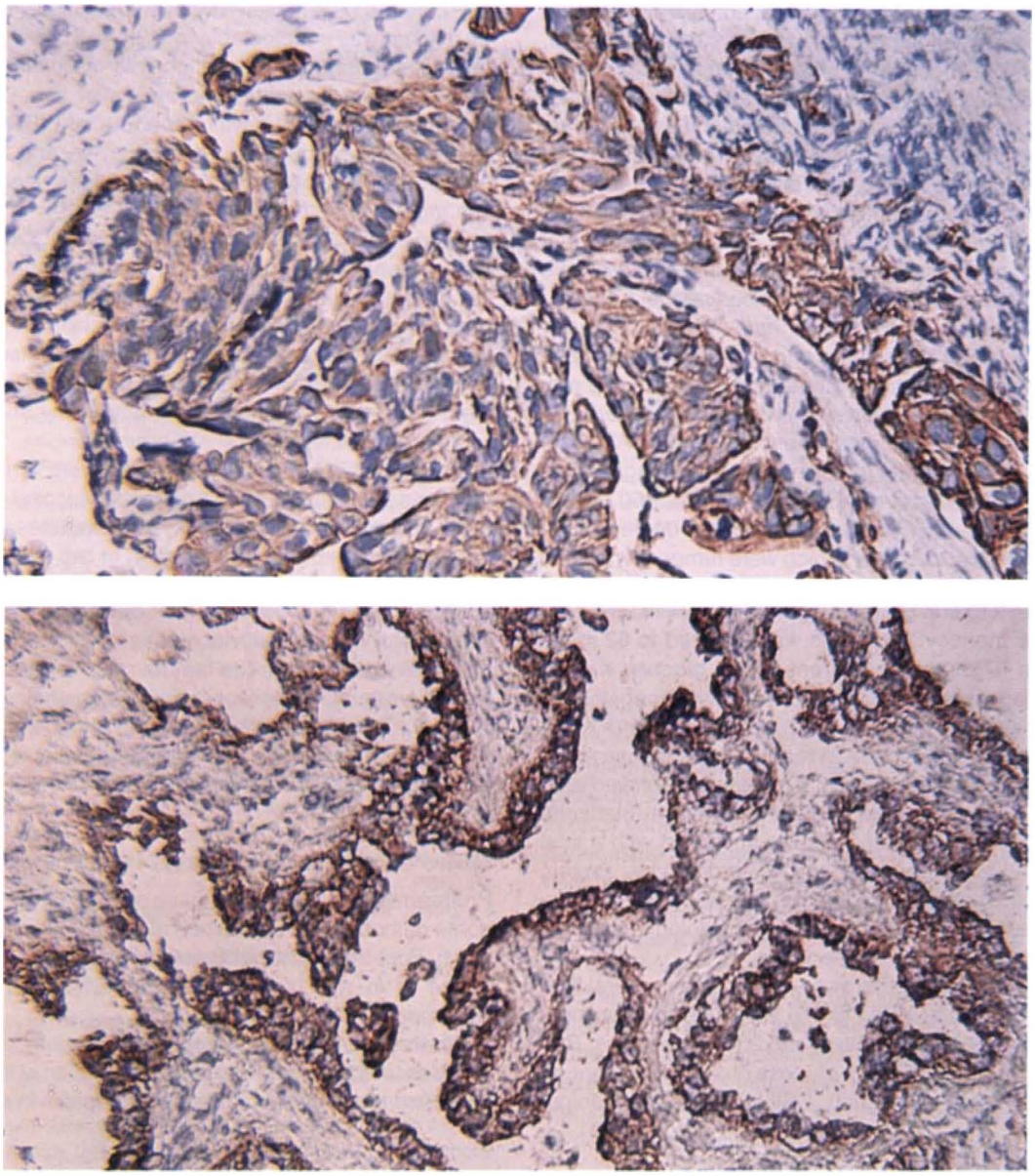

Figure 1. Carcinoma histopathology and immunoperoxidase staining. (*Top panel*) Pretreatment transbronchial biopsy shows expression of KS1/4-reactive antigen by adenocarcinoma. There is extension into the alveolar walls, and nests of malignant cells are present within alveolar spaces. Normal surrounding lung and fibrous tissue is not stained. Immunostained with MAb KS1/4. Magnification: ×45. (*Bottom panel*) Post-treatment lymph node biopsy immunostained with horse antimouse antibody and documenting that MAb KS1/4 is bound to metastatic carcinoma. Magnification: ×95.

mented intense staining of bound KS1/4 to the duodenal mucosa (Figure 2). The histopathologic features of pretreatment duodenal biopsies were normal. Post-treatment, in some patients, the villous architecture was focally flattened with a loss of goblet cells. A mild increase in lymphocytes, plasma cells, polymorphonuclear leukocytes, and eosinophils could be seen throughout the lamina propria (Figure 2).

Post-treatment duodenal biopsies were evaluated for the presence of complement activation. C3d and C4c deposition were not specifically associated with the surface mucosa and did not correlate with the site of MAb KS1/4 deposition post-treatment.

the escalating dose infusions, serum levels of MAb KS1/4 were measured at baseline before the initiation of each infusion and during the last hour of each infusion. In the first group of patients, serum levels increased with each dose escalation of antibody (in μg/ml: 50 mg, 11.6 ± 2.1 [SE]; 100 mg, 31.6 ± 5.9; 250 mg, 64.6 ± 9.8; 350 mg, 76.2 ± 6.9; 500 mg, 89.5 ± 23.3; 500 mg, 132.3 ± 27.7). In the second group of patients, serum levels increased at the second 24-h time points and then decreased or stabilized by the third time point (in μg/ml, $\bar{x}$: 350 mg/M², 75, 89, 46; 425 mg/M², 94, 129, 130). For the one patient who completed the first infusion at 500 mg/M², the maximal serum level achieved was 125 μg/ml at the termination of the infusions and rapidly

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