Annals of Surgical Oncology **15**(11):3299–3307
DOI: 10.1245/s10434-008-0070-4

The Effects of Adjuvant Experimental Radioimmunotherapy and Hyperthermic Intraperitoneal Chemotherapy on Intestinal and Abdominal Healing after Cytoreductive Surgery for Peritoneal Carcinomatosis in the Rat

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Background: Cytoreductive surgery (CS) followed by hyperthermic intraperitoneal chemotherapy (HIPEC) results in limited survival benefit and high morbidity and mortality rates in patients with peritoneal carcinomatosis (PC). Radioimmunotherapy (RIT) after CS of experimental PC has been shown to increase survival and compare favorably to HIPEC. The effects of RIT and HIPEC on wound healing after CS need to be determined.

Methods: PC was induced by intraperitoneal inoculation of CC-531 colon carcinoma cells in Wag/Rij rats. Animals were subjected to CS and anastomotic construction only or followed by RIT or HIPEC. RIT consisted of 74 MBq ^{177}lutetium-labeled anti-CC531 antibody MG1. HIPEC was performed by a closed abdominal perfusion technique using mitomycin-C during 60 minutes. Anastomotic and abdominal wall strength measurements were performed 3 and 5 days after surgery.

Results: At day 5, bursting pressure in ileum and colon anastomoses in the CS + HIPEC group, but not in the CS + RIT group, was lower ($P < .01$) than in the CS group. In the CS group, the colonic bursting site was more often outside the true anastomotic area (8 of 12 animals) than in the CS + HIPEC (1 of 12) and CS + RIT (5 of 12) groups. Abdominal wall strength in the CS + HIPEC group was significantly ($P < .01$) lower, at both measuring points, than that in both the CS group and the CS + RIT group. There was no difference between the latter.

Conclusion: As adjuvant to CS, HIPEC showed a decrease in anastomotic and abdominal wall wound strength in a model of PC of CRC, whereas RIT did not.

Key Words: Radioimmunotherapy—Cytoreductive surgery—Heated intraperitoneal chemotherapy—Peritoneal carcinomatosis—Wound strength.

Extensive surgical debulking procedures (cytoreductive surgery, CS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) are considered today's first choice for the treatment of peritoneal carcinomatosis (PC).[1] The extensive nature of the surgical procedure, where all macroscopic tumor is removed, often requires the construction of multiple bowel anastomoses.[2,3] The concomitant application of cytotoxic temperatures and high local concentrations of chemotherapeutic agents results in a median survival of 13–34 months. This way, a 5-year survival rate of 19–27% can be obtained.[4–7] However, patients suffer from high morbidity and mortality rates of up to

Published online August 19, 2008.
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50% and 8%, respectively. A substantial proportion of the morbidity consists of complications related to anastomotic repair such as anastomotic dehiscence and intra-abdominal abscess formation. In addition, enteral fistulas may develop.[2,3]

Previously, we have demonstrated in rats with PC that CS followed by adjuvant radioimmunotherapy (RIT) effectively prolongs survival[8,9] and could be even more effective than HIPEC in this respect.[10] Moreover, adjuvant intraperitoneal RIT resulted in significantly less treatment-related toxicity, as reflected in loss of body weight and the occurrence of diarrhea and clinical discomfort.

Very recently, it has been reported that HIPEC reduces anastomotic strength in the colon of healthy rats.[11] Our hypothesis is that RIT may be less detrimental in this respect. Here, we report on an experiment in rats with peritoneal carcinomatosis of colonic origin that were treated by CS and subsequent anastomotic construction in both ileum and colon. The effects of adjuvant HIPEC and RIT on the development of early wound strength in the intestine and in the abdominal wall were determined.

MATERIALS AND METHODS

Study Design

Seven days after intraperitoneal tumor induction with CC531 tumor cells, 72 rats (24 per treatment group) underwent CS, followed by the construction of anastomoses in both ileum and colon. Thereafter, animals were randomly assigned to immediately receive either phosphate buffered saline (CS group) or adjuvant HIPEC (CS + HIPEC group) or RIT (CS + RIT group). Within each treatment group, half of the animals were sacrificed 3 and 5 days after operation for the measurement of mechanical and biochemical parameters for repair. A further nine animals, three for each group, were used for histological examination of the anastomoses 5 days after surgery.

Model of Peritoneal Carcinomatosis

Male WAG/Rij rats (10–12 weeks old, body weight 240–260 g, Harlan Horst, The Netherlands) were housed under nonsterile standard conditions (temperature, 20–24°C; relative humidity, 50–60%; 12 h light/dark cycle) in filter-topped cages (two rats per cage), with free access to food (Ssniff, Bio Services Uden, The Netherlands) and water. Rats were allowed to become accustomed to laboratory conditions for at least 1 week before experimental use. Physical condition was examined daily, and total body weight was recorded daily by a biotechnician, who remained blinded to the therapeutic regimen. All experiments were approved by the local Animal Welfare Committee of the Radboud University Nijmegen and were carried out in accordance with the Dutch Animal Welfare Act of 1997.

The syngeneic rat colon carcinoma cell line CC531, originally induced in Wag/Rij rats by intravenous injection of 1,2-dimethylhydrazine,[12] was injected intraperitoneally, as previously described.[8]

Surgical Procedures

Surgery was performed under general anesthesia using isofluorane 3%, O_2 and N_2O 1:1. All rats underwent a complete midline laparotomy, followed by careful inspection of the abdominal contents for tumor growth. Tumor growth at the various intra-abdominal locations was scored as 0 (no tumor), 1 (little), 2 (moderate), or 3 (abundant). Within each animal, the sum of the tumor scores represented the peritoneal cancer index (PCI).[8] Cytoreductive surgery was performed, including an omentectomy, by radically removing all macroscopic tumor deposits. Irresectable tumors were cauterized. Subsequently, 5 mm of the distal ileum was resected, approximately 15 cm proximal to the cecum, and continuity was restored by constructing an end-to-end anastomosis with 8 single-layer, inverting, interrupted sutures (Ethilon 8-0, Ethicon, Germany). A similar procedure was then performed in the descending colon, approximately 3 cm proximal to the peritoneal reflection. Following the construction of this anastomosis, saline (3 mL) was administered intraperitoneally or a RIT or HIPEC procedure was performed. After anastomotic construction, the abdominal wall was closed in two layers using continuous Vicryl 3/0 sutures for the muscular component and iron wound clips for the skin. When followed by HIPEC, the laparotomy was closed in one layer after installing the catheters. Prior to and at the end of the procedure 10 mL of saline was given subcutaneously, for prehydration and rehydration. Thirty minutes prior to surgery and twice daily until the third postoperative day, rats were given buprenorphine (5 μg, 0.1 mL/rat/day) for analgesia.

HIPEC

The HIPEC procedure was performed as previously described.[10] Briefly, two multiperforated cath-

eters were placed through the lateral abdominal wall bilaterally and the abdomen was closed, thus creating a closed perfusion system. The perfusion system was filled with 250 mL 0.9% NaCl, containing 4 mg MMC (Mitomycin-C Kyowa, Christiaens). This perfusate was heated (inflow temperature was set at 44°C) and infused into the peritoneal cavity for the duration of 60 min. Gentle massage of the abdomen was used throughout the HIPEC procedure to achieve a uniform heat distribution within the peritoneal cavity. Following the heated perfusion, the abdominal cavity was flushed with warm (37°C) 0.9% NaCl for a period of 10 minutes. Thereafter, the continuous suture was opened and the perforated catheters were removed. Subsequently, the abdomen was closed in two layers.

Radioimmunotherapy

The murine MG1 monoclonal antibody (MAb), an anti-CC531 IgG2a monoclonal antibody that recognizes a 80 kDa cell surface antigen and localizes preferentially in tumors when injected in rats bearing CC531 tumors,[13] was purchased from Antibodies for Research Applications BV (Gouda, The Netherlands). Labeling of the antibody with ^{177}Lu was carried out as previously described.[8] The purified ^{177}Lu-MG1 preparation was diluted in PBS with 0.5% BSA to 0.4 MBq/μg (185 μg MG1/rat, radiolabeled with 74 MBq ^{177}Lu in 3.0 mL) prior to injection. RIT was applied intraperitoneally immediately after surgery.

Measurement of Anastomotic and Abdominal Wall Strength

Rats were killed by CO/CO_2 asphyxiation at either the third or the fifth postoperative day. The abdomen was opened, and, if necessary, adhesions were dissected carefully without manipulation of the anastomosis. Presence of adhesions, abscesses, or anastomotic dehiscence was recorded. The segments containing the anastomoses were then resected. Anastomotic bursting pressure was measured in succession as described previously.[14] The abdominal wall breaking strength was measured in two isolated strips (2 to 5 mm × 10 mm) from the sutured midline incision using a tensiometer.

Biochemical Analysis and Histology

After measuring wound strength, the tissue samples were cleaned from adhering tissue and debris, and 5-mm samples containing the anastomosis or the abdominal suture line in the middle were frozen in liquid nitrogen and stored at −80°C until further processing. After lyophilization, samples were weighed, pulverized, and lyophilized again. The hydroxyproline content, as a measure of the collagen content, was measured by high-performance liquid chromatography after hydrolysis with 6 M HCl and derivatization with dabsyl-chloride. Preparation of tissue extracts and procedures for quantitative gelatin zymography to measure the activity of matrix metalloproteinases (MMP) 2 and 9 have been described previously.[15]

Sections of anastomoses and abdominal wall, originating from separate animals that had not been subjected to strength measurements were stained using hematoxylin & eosin (H&E) and/or used for immunohistochemistry using the murine MG1 antibody in combination with a HRP conjugated horse-antimouse IgG antibody, (Vector Laboratories Inc., Burlingame, CA, USA). For analysis of collagen fibers, sections were stained with picrosirius red.

Autoradiography

Tissue sections containing the anastomosis or part of the abdominal wall containing the suture line were exposed to a storage phosphor imager screen for 20 minutes immediately after strength measurement at day 3.The screen was scanned in a phosphor imager system (Molecular Imager GS363, BioRad Laboratories, Hercules, CA, USA) at a pixel size of 100 × 100 μm. Images were processed with Quantity One software (version 4.5.2, BioRad Laboratories, Hercules, CA, USA).

Statistical Analysis

To analyze the differences between the three groups regarding wound strength, body weight, and MMP levels, a Kruskal-Wallis test was used, with a Dunn correction for multiple comparisons. For hydroxyproline levels, an analysis of variance (ANOVA) with a Tukey-Kramer was performed. If only two data sets were compared (e.g., data at day 3 vs. day 5 within one group) the Mann-Whitney test was used. All tests were two-sided; the level of significance was set at $P < .05$.

RESULTS

Surgical Procedures

No animals died and no complications occurred during the various treatment procedures. At dissec-

TABLE 1. *Treatment group characteristics (peritoneal cancer index; PCI) found during laparotomy before the administration of the adjuvant therapy*

	Median (range)					
	CS + PBS		CS + HIPEC		CS + RIT	
Disease characteristics	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5
Body weight Mean (range)	252 (232–274)	254 (242–266)	258 (246–270)	259 244–270	257 (248–277)	258 (243–269)
Tumor score per site						
Greater omentum	2 (1–3)	2 (2)	2 (1–2)	2 (2)	2 (1–2)	2 (2–3)
Liver hilum	1 (1)	1 (0–1)	1 (1)	1 (1)	1 (0–1)	1 (1)
Perisplenic	0 (0)	0 (0–1)	1 (0–2)	0 (0)	0 (0)	0 (0)
Mesentery	1 (0–2)	1 (0–1)	1 (1)	1 (0–2)	0 (0–1)	1 (1–2)
Gonadal fatpads	1 (0–2)	0 (0–2)	1 (0–1)	1 (0–2)	0 (0–2)	1 (0–2)
Diaphragm	0 (0)	0 (0)	0 (0)	0 (0–1)	0 (0–1)	0 (0–1)
Parietal peritoneum	1 (1)	1 (1)	1 (0–1)	1 (0–1)	1 (0–1)	1 (1)
Total	5 (1–7)	6 (2–8)	5 (2–8)	6 (2–9)	5 (4–9)	5 (2–8)

CS, cytoreductive surgery; HIPEC, heated intraperitoneal chemotherapy; RIT, radioimmunotherapy. PCI is expressed as median and range.

tion, three animals, one in the control and two in the RIT group, showed abscess formation at the site of the anastomosis, without statistical differences between groups ($P = .4$).

At the time of surgery, tumor nodules were present in the omentum, liver hilum, mesentery, and gonadal fat pads (1–3 mm diameter). Median PCI score at time of surgery was 5 (range, 1–9) and was the same in all experimental groups (Table 1). Intra-abdominal temperature during the HIPEC procedure ranged between 39.0 and 43.2°C, with a median of 42.3°C. The rectal temperature ranged between 34.1 and 34.8°C, with a median of 34.6°C.

Preoperative weight was similar in all groups. All animals lost weight, and after 5 days weight loss after CS, CS + HIPEC, or CS + RIT was $10.1 \pm 4.2\%$, $14 \pm 6.6\%$, and $12.2 \pm 5.2\%$, respectively ($P < .01$ for CS vs. CS + HIPEC). Also, animals treated with CS + HIPEC were lethargic and showed signs of physical discomfort during the first days. In addition, these animals suffered from diarrhea.

Wound Strength

The bursting pressure at day 3 showed no differences between the three groups with respect to both absolute value and bursting site (always within the anastomotic area). The average bursting pressure of the ileal anastomoses increased significantly between day 3 and day 5 in all groups ($P < .0001$, $P < .04$, and $P < .03$ for CS, CS + RIT, and CS + HIPEC, respectively), although the relative increase was lowest in the CS + HIPEC group (Fig. 1). In the colon anastomoses, bursting pressures significantly increased in the CS and CS + RIT groups ($P < .0001$ and $P < .02$, respectively) but not in the CS + HIPEC group. As a result, marked differences between groups were seen at day 5 (Fig. 2). The lowest median bursting pressure in both ileal and colonic anastomoses was found in the CS + HIPEC group and the highest in the CS group ($P < .01$). Although median values in the CS + RIT group were lower than in the CS group, these differences were nonsignificant. Loss of strength in the CS + HIPEC group was further illustrated by a shift in bursting site, particularly in the colon. Here, rupture occurred within the suture line in 4 of 12 cases in the CS group and in 11 of 12 ($P < .05$) cases in the CS + HIPEC group, while the CS + RIT group showed an intermediate value (7 of 12).

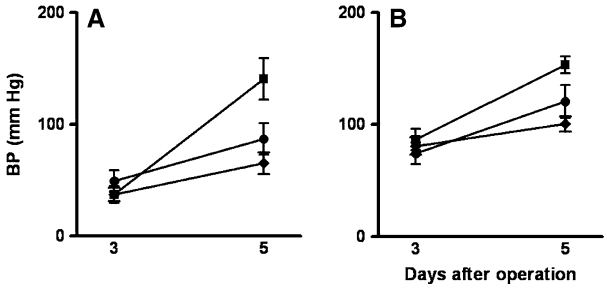

FIG. 1. Gain of wound strength in intestinal anastomoses. Mean (±SEM) of the bursting pressure in ileal (**A**) and colonic (**B**) anastomoses at 3 and 5 days after operation, for the CS (■), CS + HIPEC (♦), and CS + RIT (•) groups.

For the abdominal wall strength, significant differences were seen at both postoperative days (Fig. 3). From day 3 to day 5 the abdominal breaking strength increased approximately sixfold in all groups. Again, strength was highest in the CS group and lowest in the CS + HIPEC group: $P < .01$ at both day 3 and day 5. Also, abdominal wound

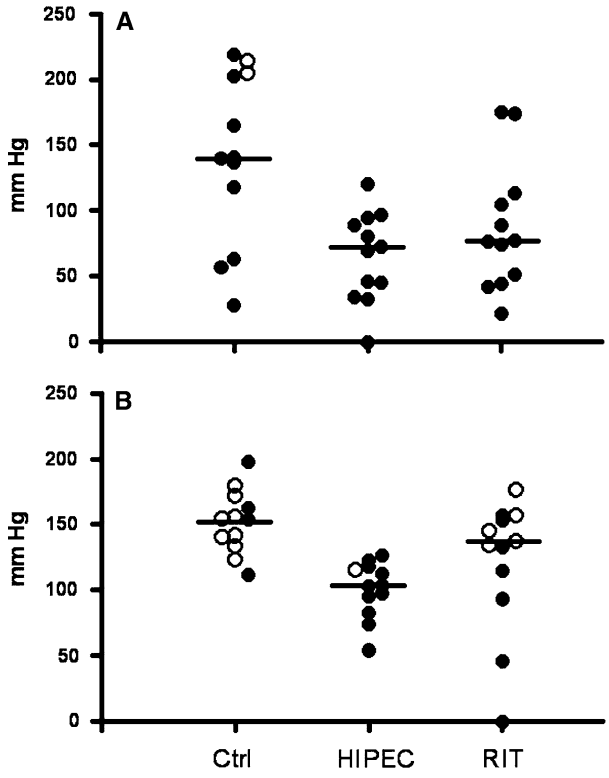

FIG. 2. Anastomotic bursting pressure 5 days after operation. Data represent individual measurements in ileum (**A**) and colon (**B**). Closed symbols denote anastomoses rupturing within the suture line and open symbols those rupturing outside the wound area. Horizontal lines give the median values.

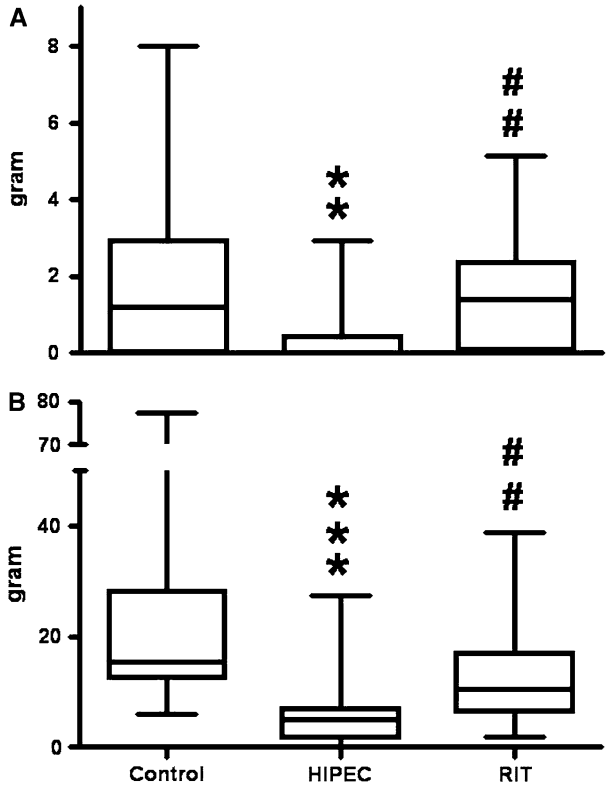

FIG. 3. Abdominal wall strength. The breaking strength is given for day 3 (**A**) and day 5 (**B**) postoperatively. Horizontal bars represent medians and vertical bars range while boxes give 25–75 percentiles. *** $P < .001$ vs. CS; **: $P < .01$ vs. CS; ##: $P < .01$ vs. CS + HIPEC.

strength was significantly ($P < .01$) lower in the CS + HIPEC group than in the CS + RIT group. The average wound strength was similar in the CS and CS + RIT groups.

Hydroxyproline Content and Gelatinase Activity

The wound hydroxyproline content showed no differences between groups at day 3 after surgery. At day 5, the average hydroxyproline content at all three sites was lowest in the CS + HIPEC group and highest in the CS + RIT group (Fig. 4). However, these differences remained nonsignificant in the colonic anastomoses. In the ileum, values in the CS + RIT group were significantly higher than in both the CS ($P < .01$) and the CS + HIPEC ($P < .001$) group, while in the abdominal wound this was only the case if compared with the CS + HIPEC group ($P < .05$).

There were no differences in proMMP-9 activity between groups. Active MMP-9 remained absent. On average, pro-MMP-2 activities were highest in the CS + RIT groups, most clearly in the intestinal wounds. More explicit differences were observed for active MMP-2 (Fig. 5). With the exception of colonic anastomoses at day 3, median values were significantly higher in the CS + RIT group than in the other groups, in some cases by far more than 100%. As a consequence, the ratio between active and inactive MMP-2 was also highest in the CS + RIT group. For instance, median values for this ratio at day 3 in ileal anastomoses were 0.19 in both CS and CS + HIPEC groups and 0.37 ($P < .001$ vs. both other groups) in the CS + RIT group.

Histology

H&E staining of sections of the ileum and colon showed an increased number of polymorphonuclear neutrophils at the anastomotic site compared with the adjacent bowel wall, indicating an inflammatory response in all groups, but no architectural differences as a result of adjuvant treatment (Fig. 6).

Picrosirius red staining of the ileum sections revealed few collagen fibers in the true wound area in all treatment groups. In contrast, in the colon anas-

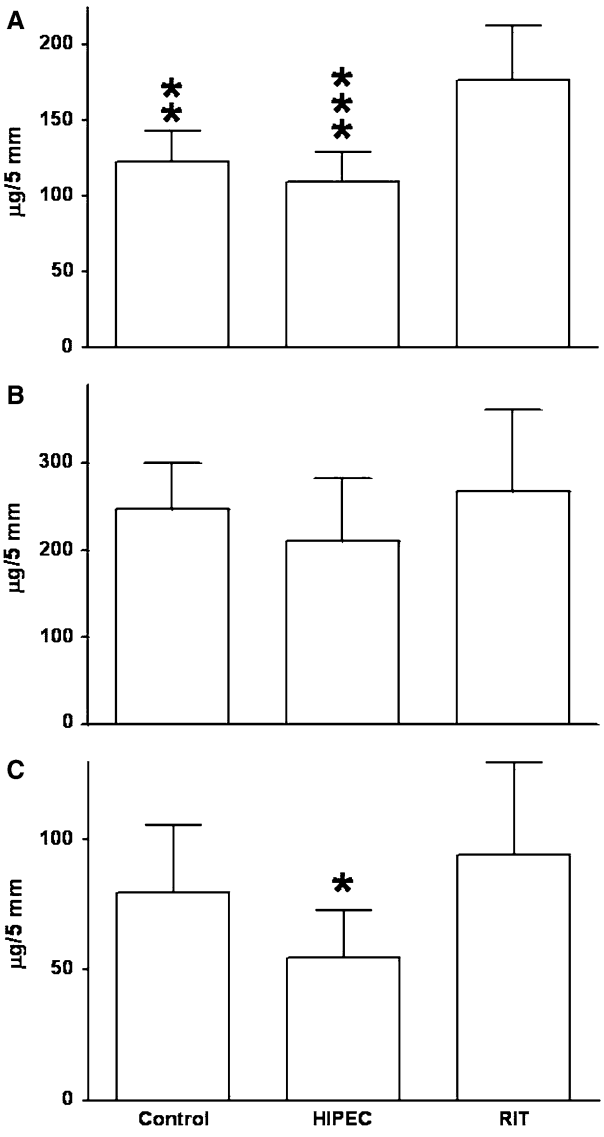

FIG. 4. Wound hydroxyproline content 5 days after operation. Bars represent mean values (+ SD) for ileal (A) and colonic (B) anastomoses and fascial wounds (C). ***, **, and *: $P < .001$, .01, and .05, respectively, vs. CS + RIT group.

tomoses there was significant collagen formation in the CS and CS + RIT groups, whereas this appeared less pronounced in the CS + HIPEC group. Sections of the abdominal wall showed collagen formation in all treatment groups.

Autoradiography

Autoradiographic analysis of the bowel anastomosis and abdominal wall sections are shown in Fig. 7. Clearly, nonspecific binding of the radiolabeled antibody to the surgical sites occurred, both at the anastomosis and the laparotomy wound.

DISCUSSION

This study demonstrates that the use of adjuvant RIT after CS did not significantly affect anastomotic strength. In contrast, the adjuvant application of HIPEC was associated with significantly decreased anastomotic strength. In addition, HIPEC as an adjuvant after CS resulted in significantly lower abdominal wall strength than adjuvant RIT.

Morbidity after cytoreductive surgery followed by heated intraperitoneal chemotherapy is related to surgical/anastomotic complications in 35–54% of the cases.[2,16,17] Moreover, the number of suture lines constructed is an independent variable linked to morbidity, suggesting that an increased number of anastomoses is associated with a growing chance for anastomotic leakage.[18] A substantial part of anastomotic-related complications stems from small-bowel perforation or anastomotic leakage.[2,3,17] Also, not only morbidity, but also mortality is linked to anastomotic-related complications. Patients may succumb to peritoneal sepsis caused by intra-abdominal abscess formation related to anastomotic leakage.[19] Therefore, the focus of new adjuvant treatment modalities should not be aimed at improving disease-free survival alone but also at reducing perioperative morbidity and mortality.

Few preclinical studies on the application of HIPEC or the intraperitoneal administration of MMC have been reported. HIPEC and MMC have been associated with marked toxicity in the form of lethargy, marked weight loss, and bacterial translocation.[20] It is well known that intraperitoneal cytostatics can severely affect anastomotic strength.[21] This has also been suggested to be the case for MMC, although in the most recent report anastomoses were analyzed at the 10th postoperative day, when the bursting pressure no longer reflects actual wound strength.[22] Makrin and colleagues performed a HIPEC procedure in rats, using MMC in a concentration of 0.02 mg/mL in 200 mL of perfusate (total dose 4 mg), circulating for 20 minutes at a temperature of 40°C.[23] The anastomosis consisted of a subtotal dissection (70–80% of the circumference) of the cecum. Thus, the experimental setup deviated significantly from the clinical situation (incomplete anastomoses, short perfusion time). The investigators found a decreased bursting pressure in the MMC group compared with controls. The most recent study on anastomotic strength and HIPEC was performed by Pelz and colleagues.[11] Here, HIPEC was also performed with MMC (20 mg/m^2, intraperitoneal temperature 40.5–41°C), either before or after anas-

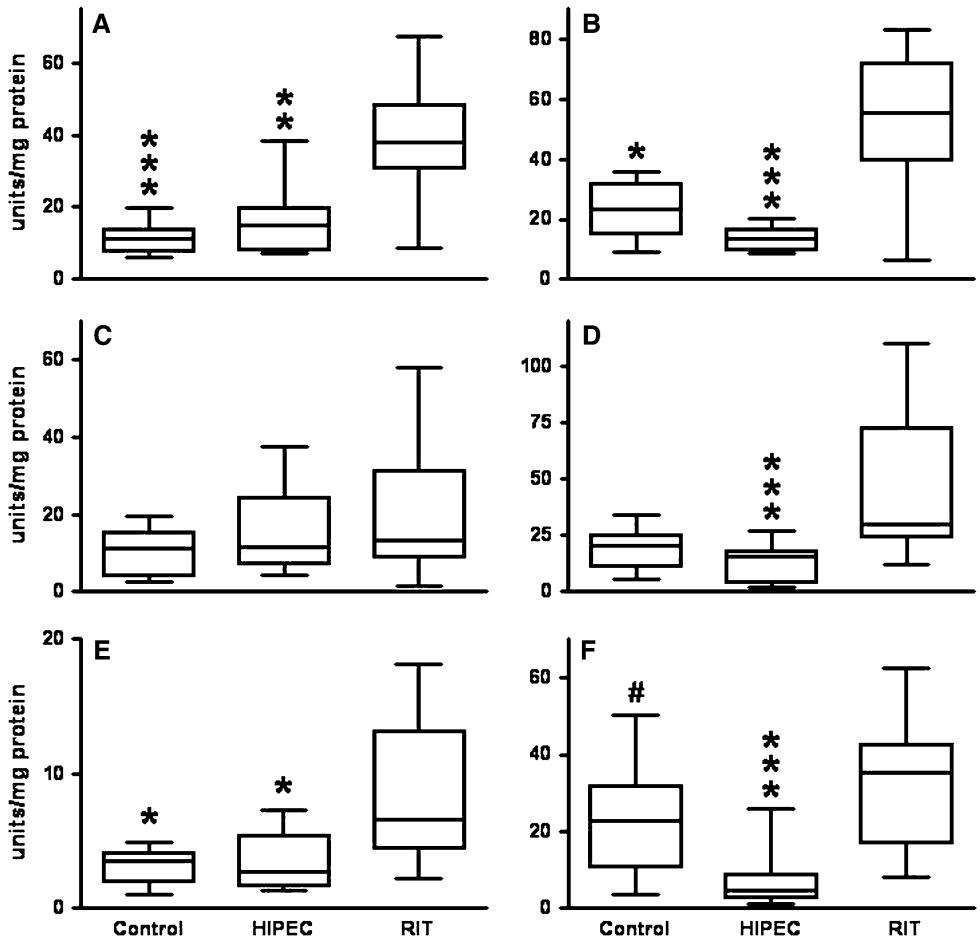

FIG. 5. Active MMP-2 in wounds. Specific activity is given at both 3 (**A**, **C**, **E**) and 5 (**B**, **D**, **F**) days after operation in ileum (**A**, **B**), colon (**C**, **D**), and fascia (**E**, **F**). Horizontal bars represent medians and vertical bars range while boxes give 25–75 percentiles. ***, **, and *: $P < .001$, .01, and .05, respectively, vs. CS + RIT group. #: $P < .05$ vs. CS + HIPEC group.

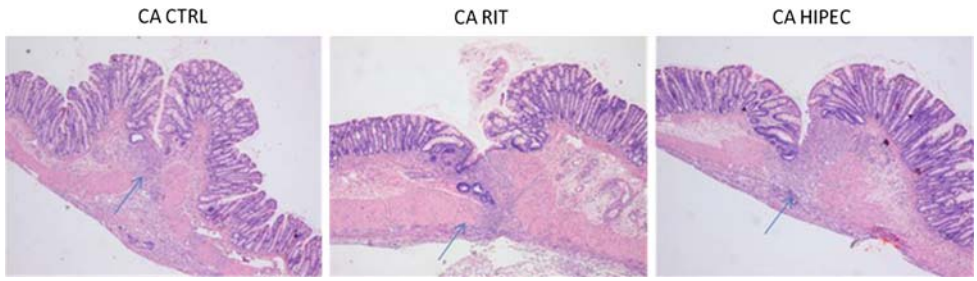

FIG. 6. Representative H&E stained slices of colon anastomoses (CA) 5 days after surgery for the respective treatment groups. Arrows indicating the anastomoses.

tomotic construction in the colon. In both cases, HIPEC significantly reduced the anastomotic bursting pressure, as measured both 4 and 10 days after surgery. However, so far all studies on the effects of HIPEC on wound repair in a preclinical setting have been performed in healthy rats lacking the preceding tumor growth and cytoreductive surgery applied in the present study. Moreover, in our study anastomoses were constructed in those parts of the bowel that in the clinical setting are prone to be resected during surgical debulking procedures (ileum and sigmoid colon). Thus, the present experiment more

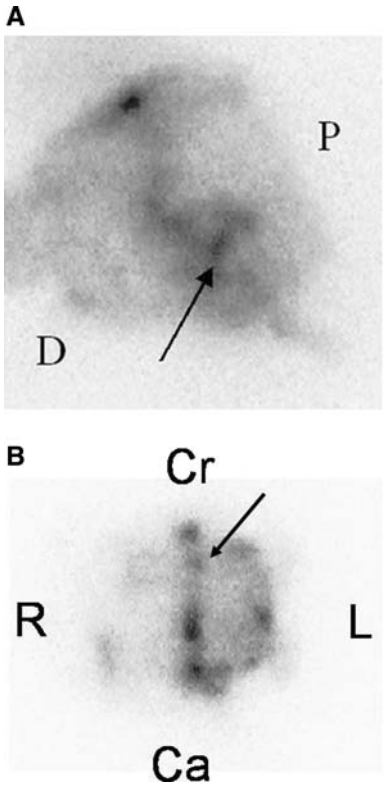

FIG. 7. Autoradiography of surgical wounds. A longitudinal cross section of an ileal anastomosis (**A**) showing uptake of the radiolabeled antibody at the surgical site (arrow). Autoradiographic images of an abdominal wall (**B**) containing the laparotomy wound, showing uptake of the radiolabeled antibody at the surgical site (arrow). CR indicating the cranial part, CA indicating the caudal part of the wound, R indicates the right side, L indicates the left side of the animal.

closely resembles the clinical situation than any of the experiments reported so far.

In our previous report on the comparison of the adjuvant use of RIT versus the adjuvant application of HIPEC, we showed that adjuvant RIT was capable of achieving a significant improvement in survival compared with CS alone in a model of peritoneal carcinomatosis of colonic origin in rats. In contrast, the increased survival after adjuvant HIPEC remained nonsignificant.[10] In addition, the treatment-related toxicity was highest in the CS + HIPEC group. So far, there have been no reports about complications in healing of wounds in the intestine or abdominal wall after intraperitoneal RIT. The reason for this might be the fact that in clinical trials adjuvant RIT is administered weeks after any surgical procedure. At that time, anastomoses (and laparatomy wounds) are already strong and less prone to disruption. However, we have also demonstrated that the optimal moment, in terms of maximal effect on survival, for administration of adjuvant RIT is immediately after cytoreductive surgery.[9] The only preclinical data available on wound healing and irradiation regard the results of intraoperative external beam radiation therapy. In this setting, even moderate radiation doses delayed the healing of colonic anastomoses.[24] There have been, as yet, no reported data on the potential effects of low dose radioimmunotherapy on (intestinal) wound healing.

Although the bursting pressure of both anastomoses at day 5 after surgery was lowest in the CS + HIPEC group, there was no significant difference in hydroxyproline content with the CS group. The same was true for the abdominal wall at both days after surgery. Thus, the hydroxyproline content in the segments containing the wound does not correlate with wound strength. This might be due to the fact that those segments contain an excess of uninjured tissue and thus are not very specific for the true wound area. On the other hand, it may also mean that a defect in collagen quality rather than quantity leads to the loss of wound strength in the CS + HIPEC group. Active MMP-2 levels were highest in the CS + RIT group, but this did not lead to reduced wound strength. Apparently, the presence of enhanced MMP-2 activity does not lead to a degree of matrix degradation that would be sufficient to affect wound strength. A rise in active MMP-2 and MMP-9 after radiation was also reported by Strup-Perrot after external beam radiation of the colon in rats.[25]

A possible explanation for the presence of antibody at the site of the anastomosis, as observed by autoradiographic imaging, could be nonspecific binding. Nonspecific antibodies such as IgG have been used in infection imaging. The nonspecific localization of antibodies in inflamed tissues is caused by the enhanced vascular permeability in these tisssues.[26] This would then result in antibody accumulation at sites of vasodilation (surgical injury) as can be seen by autoradiography. However, the suggested nonspecific binding was not associated with decreased wound strength in the present study.

From the present data, we conclude that the use of RIT as an adjuvant to cytoreductive surgery is a treatment with low toxicity that does not significantly impair wound healing. In contrast, today's first choice of adjuvant treatment, HIPEC, is associated with a marked reduction in anastomotic and abdominal wall strength. These data therefore provide further justification for clinical studies using intraperitoneal RIT postoperatively in case of peritoneal carcinomatosis of colorectal cancer.

ACKNOWLEDGMENTS

The authors wish to thank Henny Eikholt, Maarten Brom, and Bianca Lemmers (Radboud University Nijmegen Medical Center, Central Animal Laboratory) for their assistance in the animal experiments and Annemarie Eek for the excellent assistance on radiolabeling procedures.

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