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The effect of preoperative high-dose methotrexate and leucovorin rescue on the incidence of wound infection in the rat

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THE EFFECT OF PREOPERATIVE HIGH-DOSE METHOTREXATE AND LEUCOVORIN RESCUE ON THE INCIDENCE OF WOUND INFECTION IN THE RAT

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Robert Lawrence Kraft

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REVIEW OF THE LITERATURE

Historical Background

The treatment of head and neck cancer has undergone a long evolution. Prior to the turn of the century, surgery was the mainstay of therapy. Though many of today's routine resections were considered too extensive for that time, the first total laryngectomy for cancer of the larynx was performed by Billroth in 1873.[1] The discoveries of x-rays by Roentgen in 1895 and radium by the Curies in 1898 ushered in a new era in the treatment of cancer patients.

In the early 1900's, Crile observed that greater than 99% of all head and neck cancer patients died from primary disease or regional metastases. This led him to perform the first radical block dissections of the lymphatics of the neck. The three-year disease free interval of his patients increased 25%.[2] Operative mortality was so prohibitive, however, that he limited these resections to cases where the primary was in the floor of the mouth and gum.[3] With the development of 200 kv x-rays in the 1920's, investigators began to appreciate the difference in radiosensitivity of many tumors, noting that some tumors responded better to surgery while others responded better to irradiation.[4]

The first cure of advanced laryngeal cancer by therapeutic radiation was achieved by Regaud, Coutard, and Houtant

in 1922.[5] In the 1930's, Coutard developed the technique of administering radiation in fractionated doses over a period of weeks to decrease the local effects. This encouraged further use of radiation therapy for head and neck cancers. Martin utilized Coutard's technique to irradiate both primary and node-bearing areas, and supplemented the external radiation with implantation of radon seeds. Of greater significance was the fact that Martin then performed a partial or radical neck dissection for persistent disease in the neck, signalling the beginning of combined modality therapy. Although supervoltage radiation therapy had developed by this time, it was not met with overwhelming enthusiasm.[2, 5] However, in 1934, the publication of a dosage system for gamma-ray therapy by Paterson and Parker[6, 7] led to the standardization of all subsequent radiotherapy.

The 1940's, particularly after World War II, saw the development of a number of general surgical advances which made many radical head and neck procedures safer. These developments were: sulfa drugs and antibiotics available for civilian use, intravenous sodium pentothal anesthesia, endotracheal intubation, blood banks, and the harvesting of split thickness skin grafts.[3] Baclesse extended the course of radiation therapy from 3 weeks to 6, 8, or 10 weeks, thereby further decreasing the amount of acute edema and mucosal reaction.[2]

In the 1950's there was a resurgence of interest in combined radiation and surgical therapy.[8] New and more powerful

sources of radiation were introduced (e.g. Cobalt-60, betatron) to provide large doses to deeper tissue while sparing the degree of radiation to skin. Despite an increasing number of clinical trials employing chemotherapeutic agents, accepted therapy has principally remained radiation and surgery.

Cancer of the head and neck region continues to be a significant problem to diagnose and treat. In 1976, an estimated 21,400 people died from cancer of the head and neck, while approximately 66,000 new cases were reported.[9] Many of these people will be treated with a combination of radiation and surgery, a modality that has been approached with caution because of its attendant morbidity during the postoperative period.

Complications of Preoperative Irradiation

One of the major complications of operating on previously irradiated tissue is impaired wound healing. Prior to 1950, the risk of postoperative wound complications made the utilization of this combined therapy undesirable, if not prohibitive.[8] By the late 1950's, however, improvements in both radiological and surgical techniques renewed interest in this modality and prompted investigators to feel that the results outweighed the attendant risks. As reports of the results of head and neck cancer treatment with preoperative radiation filled the surgical literature, it became evident that wound complications still accounted for a major portion

of the postoperative morbidity.

Goldman et al.[10] reported on 23 patients with stage III cancer of the larynx and laryngopharynx. The patients received 5500 rads over 5 weeks, then underwent laryngectomy with unilateral or bilateral radical neck dissection between 3 and 6 weeks after radiation. There were 29 procedures performed, resulting in 13 wound complications (45%), which consisted of 8 fistulae, 3 infections, and 2 flap necroses. Wounds with fistulae took twice as long to heal as wounds without fistulae (an average of 34 days as compared to 17 days). As a result of this high complication rate, measures were instituted to attempt to reduce the development of fistulae. The next five patients were treated with neomycin mouthwash, systemic antibiotics, several days of preoperative antiseptic scrubs to the operative site, wound and nasopharyngeal suctioning, controlled expectoration of saliva, and an oxygen tent; none of these five subsequent patients developed a fistula. Although this is meaningless because of the small number of patients, it is clear that contamination of the wound, particularly from the saliva, was regarded as an important factor in the development of fistulae.

Habel[11] reviewed 463 patients with head and neck cancer who had been treated either by combined preoperative radiation and surgery (172) or by surgery alone (291) during the period from 1953-1963. The average radiation dose was 6060 rads; the operations were grouped as either radical neck dissection, laryngectomy alone, or laryngectomy with radical neck, and

the severity of complications in irradiated and non-irradiated patients was analyzed in each group. In the absence of complications, no difference was found in healing time between irradiated and non-irradiated patients. However, postoperative complications in the irradiated group led to an increase in healing time of 50% in the radical neck dissection group and 100% in the laryngectomy group. There was an overall increase in the complication rate among irradiated patients, as well as an increase in the severity of their complications; there were 5 carotid ruptures (9.7%) and 3 deaths (6%) in the group that had undergone laryngectomy and radical neck dissection after radiation, compared to 1 death from carotid rupture (1.3%) in the non-irradiated group. It was also noted that all the carotid ruptures occurred following wound infection. The author felt that direct salivary contamination predisposed previously irradiated wounds to the development of wound infections and subsequent serious complications.

Parnell[12] reported on 12 patients with advanced squamous cell carcinoma of the larynx who underwent total laryngectomy and radical neck dissection 3 to 6 weeks after a course of 5000 rads given over 5 weeks. In comparison to an equal number of randomized patients who underwent surgery alone, the irradiated patients had a markedly increased complication rate. Infection, flap slough and fistulae were noted to occur commonly in the presence of Pseudomonas. Mouth secretions were implicated in the wound contamination, and the possibility was raised of the benefits of neomycin mouthwash

without any supporting data.

In 1970, Goldman et al.[13] reported on their cumulative experience of 53 patients with cancer of the larynx and laryngopharynx treated with preoperative radiation. While no mention is made of preoperative antibiotic mouthwash or systemic antibiotics, the patients were treated with postoperative penicillin and streptomycin. The incidence of complications was 46% (41/90 operations), including 19 fistulae, 10 wound infections, and 3 flap necroses. The authors concluded that "preoperative radiation probably increases the surgical and postoperative complications in patients with cancer of the larynx and laryngopharynx," while tenaciously clinging to the belief that their "preventive" measures had "reduced complications to a level which is acceptable and more than compensated for by the greater survival rates achieved with combined therapy."

Smits et al.[14] studied 193 patients with oral and laryngopharyngeal carcinomas who had undergone irradiation with 4400-4600 rads over 4 to 5 weeks followed by en bloc resection and radical neck dissection 4 weeks later. The rate of wound infection in this study was 21%. Additional complications reported were: fistulae, 19%; flap necroses, 12%; carotid rupture, 3%. These were usually preceded by wound infection. The authors were convinced that salivary leakage into the wounds and the flaps predisposed to the formation of fistulae. Aggressive maintenance of good oral hygiene was considered extremely important; full mouth tooth extrac-

tions were performed 5 to 7 days before commencement of radiation therapy. Prophylactic antibiotics were administered, careful closure of the wounds, wound suction, and nasogastric tube feedings were employed. A year by year analysis revealed a decrease in the postoperative complication rate from 45% in 1967 to 29% in 1971.

Backstrom et al.[15] reviewed 35 patients with carcinoma of the tongue who had been given preoperative radiation with doses ranging from 3000 to 7000 rads, followed by surgery in 4-13 weeks. While no difference could be noted in the complication rate among the patients with respect to the time interval between radiation and surgery, increasing doses of radiation were associated with an increasing frequency of complications. No infections, fistulae, or mucosal or bone necroses occurred in the 9 patients receiving less than 4000 rads.

A similar observation between the dose of radiation and the frequency of complications in radical neck dissection was noted by Yarington et al.[16] The incidence of major complications in patients who had been irradiated with "curative" doses (3500 to 11,800 rads) was 44% (4/9), as compared to 29% (2/7) among those who had received planned preoperative radiation (3500 to 4700 rads) and 12% (7/61) among patients undergoing surgery alone. The overall complication rate was 26%, most of which occurred in patients who had radical neck dissection in combination with oral or pharyngeal resections.

More recently, Lawrence et al.[17] reported on 143 pre-

viously untreated patients with stages II-IV squamous carcinoma of the oral cavity, oropharynx, or pharynx. Sixty-nine patients received 1400 rads in two equal fractions 48 and 24 hours prior to surgery; all the patients had resection of the primary lesion in continuity with a radical neck dissection. The incidence of postoperative complications was 24% (17/69) for the irradiated group and 20% (15/74) for the non-irradiated group. Though the figures might indicate that a preoperative dose of 1400 rads does not lead to a significant increase in the postoperative morbidity in selected patients, the study showed neither a decrease in the local recurrence rate nor an increase in survival with this dose of radiation.

Clinical experience over the years, then, reveals that patients with head and neck cancer are at a high risk for complications, particularly wound infection, when they undergo radiation therapy prior to surgery. Furthermore, salivary contamination has been implicated as a prominent source of this infection. Shannon et al.[18] have shown that salivary flow in patients undergoing radiation therapy (mean dose 4432 rads) decreases over 6 weeks to 5% of control levels. Since saliva plays an important role in suppressing the normal microflora of the mouth, this effect leads to an alteration of the flora and an increase in the number of pathogenic species.[19] Such an alteration in oral contents might be especially virulent to a healing suture line. Ariyan[20] has shown that radiated tissue cannot tolerate such bacterial contamination as well as normal tissue. In a standard labora-

tory model, rats were given single-dose biological equivalents of 2300 rads over 2½ weeks, 4000 rads over 4 weeks, and 6000 rads over 6 weeks. The animals were then wounded at various intervals following irradiation, and their wounds innoculated with 10^4 S. aureus. The rate of infection (defined as a bacterial count of 10^5 or more per gram of tissue) increased with increasing radiation dosage. There was also a significant correlation between incidence of infection and the interval between irradiation and wounding.

Rationale for Preoperative Irradiation and an Alternative

In light of the problems with preoperative irradiation, the use of postoperative irradiation might be advocated. However, the theoretical rationale behind preoperative irradiation needs to be considered. One reason for failure of head and neck cancer surgery is local recurrence. Recurrence rates may be as high as 40-45%.[21, 22] Local recurrence may result from any or all of a number of factors: 1) inadequate removal of tumor from primary or nodes, 2) local metastasis outside the surgical margins, and 3) new primary tumor in the area. A fourth possible cause of local recurrence may be spillage of tumor cells into the wound during excision. In 1906, Lawrie wrote:

> "Where the original tumour has been removed entire inside a mass or surrounding tissue, like a core of paper in a ball of worsted, there will be no possibility of transplantation, but where the tumour is broken into during an operation the risk of transplantation must be very great. In such a case small portions conveyed by the fingers or knife

> might easily find a suitable position on the raw surface for renewed growth, hence the so-called recurrence." 23

However, the theory remains extremely difficult to prove.

Smith et al.[21] collected washings from the wounds of 120 patients with primary operable cancer (all sites) with cytological examination revealing definite evidence of tumor in 26% of the cases, cells of a suspicious nature in 14%, and no evidence of tumor in 60%. Long-term follow up of these patients[24] revealed no significant differences among the three groups as to local recurrence rate. There was, however, a significant correlation between local recurrence and development of distant metastases, suggesting that seeded tumor cells may get into the venous or lymphatic circulation. In a similar study of 69 patients with epidermoid carcinoma of the head and neck,[25] the percentages of washings positive, suspicious, and negative for tumor cells were almost identical (26, 13, and 61, respectively). The ratio of positive to negative washings was greatest when just a wide local excision of the tumor was performed, less after en bloc resection including the lymph nodes, and least when a radical neck dissection was performed alone. Nevertheless, no significant correlation could be found between presence or absence of tumor in the wound washings and local recurrence. Smith's work reportedly showed that washing with saline did not prevent tumor implant growth in experimentally seeded wounds.[21] Thus, the absence of tumor cells in a wound washing does not preclude seeding during the operative procedure.

Ideally, it would be better to prevent any wound seeding and to kill residual tumor before postoperative scarring contributed to a decrease in radiosensitivity of the tumor cells,[8] or before they had the chance to spread to a distant site. It is believed by some that preoperative radiation alters tumor cells such that seeding during surgery would not result in implantation. To the proponents of this theory, an alternative therapy is necessary if preoperative radiation were to be abandoned because of its high complication rate. It is possible that treatment with methotrexate may fulfill this role.

The clinical efficacy of methotrexate (MTX) in head and neck cancer therapy was first demonstrated in 1959 by Sullivan *et al.*[26] Ten of 18 patients showed objective tumor regression after continuous intra-arterial MTX infusion concomitant with intermittent leucovorin administered intramuscularly. Although there are a variety of treatment dosages, MTX is still considered by many to be the most effective single chemotherapeutic agent for cancer of the head and neck.[27]

Methotrexate (4-amino-N^{10}-methyl-pteroylglutamic acid; amethopterin) is a folic acid antagonist. It prevents the reduction of dihydrofolate to tetrahydrofolate by competitively binding to the enzyme dihydrofolate reductase. In so doing, it blocks thymidylate biosynthesis, effectively preventing incorporation of endogenous thymidine into DNA. Therefore, it is cell cycle-phase specific, damaging cells

in the "S" phase of division; presumably, its action is enhanced in rapidly dividing cell populations. Leucovorin (N^5-formyl-tetrahydrofolate; folinic acid; citrovorum factor) bypasses the block imposed by MTX, because its metabolism to tetrahydrofolate does not require dihydrofolate reductase. In addition, leucovorin competes with MTX for uptake into cells, and it displaces intracellular MTX. Its value as a protective agent depends on most of the normal cells of the body being in the "G_o" (resting) phase, and, hence, not susceptible to the action of MTX.[28, 29, 30, 31]

Methotrexate may be administered in high doses either intravenously or intra-arterially. The preferred route of administration is intravenous, because intra-arterial infusion is attended by complications of the catheterization process (i.e. cerebrovascular accidents), and because frequently the tumors extend beyond the area of infusion.[28, 32] Intravenous infusion is commonly administered over a 24 hour period, with leucovorin "rescue" commencing at the end of the infusion. Methotrexate is almost entirely excreted in the urine in its original form.

In order to justify the use of MTX as part of a combined modality approach to the treatment of head and neck cancer, it must be shown that preoperative administration of the drug would produce fewer or less severe complications than does preoperative irradiation. The major complication of preoperative irradiation is impaired wound healing, particularly due to infection and its sequelae. The question that

must be asked is whether MTX affects wound healing and, in particular, whether the treated tissues have a decreased tolerance to bacterial contamination.

Experimental Evidence of the Effect of MTX on Wound Healing

In 1962 and 1964, papers by Kiehn, DesPrez, and Benson[33, 34] referred to investigations of the effect of MTX on wound healing in mice. Their conclusions were that wound healing was not significantly affected until the systemic dosage exceeded the LD_{50}, and that one week or more should intervene between completion of infusion and surgery. The wound healing data from these studies, however, were never published.

In 1965, Calnan and Davies[29] examined the effect of various low-dose schedules of MTX on the tensile strength of wounds in rats (see Tables F through H in the appendix). Methotrexate was administered intraperitoneally. A dose of 0.375 mg/kg/day was given for 5 days postoperatively. At 5 days after wounding, tensile strength was significantly decreased to 67% that of controls ($p < 0.05$). When differing doses were given (0.125, 0.25, 0.5, and 1.0 mg/kg/day for 5 days postoperatively, the tensile strength of five-day-old wounds decreased with increasing dose, significantly so at greater than 0.3 mg/kg/day. A study of pre- and postoperative MTX given at a dose of 0.05 mg/kg/day showed that, while 5 days of postoperative treatment caused a significant decrease in the tensile strength of 5-day-old wounds ($p < 0.05$), tensile strength was least affected by 5 days of preoperative therapy.

The effect of leucovorin "rescue" was also studied. Methotrexate was administered at a dosage of 0.5 mg/kg/day postoperatively, both alone and with leucovorin 2.5 mg/kg/day. Methotrexate alone significantly depressed tensile strength at 3 days and 7 days ($p < 0.01$), but leucovorin prevented this completely. At that dosage, leucovorin alone produced significantly increased tensile strength at 3 days ($p < 0.01$), but by 5 days the effect was no longer significant.

These experiments were performed at a time when the maximum dose for a 60 kg adult was 1.5 mg/kg/day by arterial infusion, and 0.15 mg/kg/day by mouth. Leucovorin was considered to be effective only if given within 4 hours of MTX administration. It is difficult to assess how the dosage in rats compares to that in humans, but the LD_{10} for rats receiving a 5-day course of MTX I.P. has been calculated to be 0.4-0.6 mg/kg/day.[35] Significant depression of tensile strength, then, occurred with administration of about one tenth the LD_{10} postoperatively. The effect of varying preoperative dosage was not studied, and there was no mention of any wound infections.

In 1975, Cohen et al.[36] investigated the effect of MTX on the breaking strength of wounds in mice. Methotrexate was administered intraperitoneally as a single dose of 80 mg/kg (in this study, equivalent to 0.6-0.8 of the LD_{10} in BDF_1 mice) immediately after wounding. Breaking strength was measured at 3, 7, and 21 days (see Table I in the appendix). At 3 days, wound breaking strength was significantly impaired

($p<0.05$), but by 7 days wound strength had returned to control levels, where it remained through 21 days. None of the animals developed wound infections.

The aforementioned two studies employed different methods of measuring wound integrity, tensile strength and breaking strength. Tensile strength cannot be assessed with validity in the first week postoperatively, as tensile strengths during that time tend to be too small for accurate measurement. Breaking strength would appear to be a more valid measurement, especially if one were interested in the wound's potential for dehiscence. Unfortunately, since Cohen did not test a variety of doses and dose schedules, his experimental design has little correlation to clinical use of MTX.

It is clear that MTX has a deleterious effect on wound healing, even in low doses, and particularly when administered postoperatively. One has no way of knowing whether much higher doses than those tested would lead to further decreased wound healing or other wound complications. The clinical significance of these observations with regard to head and neck cancer surgery would depend not only on the dosage and time of administration relative to sugery, but also on the presence of contamination from oral contents, thought to be the major causative factor in wound complications in previously irradiated patients.

Surgical Complications of Preoperative Methotrexate

In 1962, Kiehn et al.[33] reported on a series of patients treated with intra-arterial MTX infusion. The MTX dose was 50 mg/day for 6-7 days, with concomitant injections of leucovorin 6 mg I.M. every 6 hours. Systemic complications included leukopenia in some patients (one patient developed severe leukopenia and died of fulminant pneumonitis). However, local complications dominated the picture, developing in approximately 50% of the patients who were treated for palliation by infusion alone. Nine patients were treated with combined chemotherapy and surgery; wound complications, consisting of wound breakdown, separation, and infection occurred in 5 patients who were operated on within two weeks following completion of the infusion. In one patient, infection following radical neck dissection led to carotid rupture. No wound complications occurred in 2 patients who underwent surgery 3 and 6 weeks, respectively, following infusion, or in 2 patients whose surgery preceded infusion by 2 weeks.

Kiehn's clinical impression was that "the drug had a noxious effect on the wound." The high incidence of infections and breakdown of catheter sites was felt to be secondary to the high concentration of the drug. The local complications made subsequent neck dissections more difficult. With the catheter placed in the temporal artery, wound complications were reduced.

In 1964, DesPrez et al.[34] reviewed 13 patients with

head and neck cancer who had been treated with preoperative MTX, 50 mg/day for 7-10 days by intra-arterial infusion (temporal artery) with concomitant leucovorin 6 mg I.M. every 6 hours, and surgery performed at an unspecified time afterward. Wound complications included 4 fistulae and 1 wound separation (38%). The wound separation occurred in a patient who was operated on the day following completion of his infusion.

In 1975, Tarpley et al.[37] reported on a series of 30 patients with epidermoid carcinoma of the head and neck. They received an intravenous MTX infusion of 240 mg/m^2 over 24 hours, followed by leucovorin, 75 mg I.V. over 12 hours, then 12 mg I.M. every 6 hours for 4 doses. Two days later, this regimen was repeated. Surgery was performed 5-8 days following completion of the second infusion. Systemic toxicity was not severe. Postoperative complications included 3 wound infections, 2 orocutaneous fistulae, 4 pneumonias, and 13 fevers greater than 38°C. When compared to an equal number of retrospectively selected controls, no increase in postoperative morbidity was demonstrated. However, studies comparing retrospective controls are not very valid or revealing.

Arlen[38] employed both MTX and radiation preoperatively in the treatment of 50 patients. A fractionated course of 2000 rads was delivered to the primary site and node-bearing areas. After an unspecified interval, MTX was administered intravenously at a dose of 240-500 mg/m^2 over 24 hours.

Leucovorin was then begun with 75 mg I.V. over 12 hours, then 25 mg I.M. every 12 hours for 4 doses. This regimen was repeated. Surgery was performed 3-5 days after completion of the second infusion. Six patients developed severe stomatitis, but Arlen denied any interference with wound healing by the preoperative therapy. However, he did not report his complications. Regardless of whether MTX or radiation were responsible for wound complications, the lack of controls or statistical analysis makes evaluation of his claim difficult.

In summary, laboratory investigations of MTX administration has shown a significant effect on wound healing, even in small doses; larger doses may show greater effects. The literature on clinical experience indicates that after a high-dose infusion of the drug, infection is a major component of postoperative wound complications in head and neck cancer patients. Though the clinical studies dealing with high-dose intravenous infusion do not suggest that morbidity is increased, as do earlier studies, their techniques leave certain questions unresolved, and, as such, cannot be properly evaluated at this time.

Kiehn commented that "factors other than infusion of the drug may have influenced the development of the complications,"[33] yet no one has attempted to identify any contributory factors, other than "technique."[32] In light of the complications with preoperative irradiation discussed above, it would be logical to ask whether MTX causes decreased resistance to bacterial contamination of a surgical wound. A

well controlled clinical investigation in head and neck cancer patients would be difficult to achieve, as a result of the great variability in presentation of the tumors, the effects of previous unsuccessful therapy, and the presence of an abundant microflora within the oropharynx. Therefore, a good laboratory model is necessary. This study examines the effect of high-dose MTX on the ability of wounds to tolerate bacterial contamination. It further evaluates the optimal time that may be necessary between treatment and wounding to achieve normal healing.

In selecting the dosage of MTX, the intent was to administer a dose large enough to kill all the rats, yet not so lethal as to prevent rescue by leucovorin at 24 hours. It was also hoped that serum MTX levels would approach the "therapeutic range" (10^{-5}M)[39], albeit transiently. Our preliminary work established that 300 mg/kg of MTX was lethal to 50-100% of the rats, and that mortality was reduced to 30% after 100 mg/kg of leucovorin administered at 24 hours. Higher doses of MTX were more consistently lethal, but leucovorin could not rescue sufficient numbers of rats to make use of these doses feasible.

MATERIALS AND METHODS

Animals

One hundred twenty-eight 6-week-old, male, Sprague-Dawley rats (Charles River Laboratories, Kingbridge, Mass.), weighing 165-197 g (average 181.4 g), were used. Rats were fed a standard laboratory pellet diet and tap water ad libitum.

Drugs

Methotrexate (sodium salt) and leucovorin (calcium salt) were obtained from the National Cancer Institute, Drug Synthesis and Chemistry Branch. Methotrexate was stored in a refrigerator, and leucovorin was deep-frozen at -80°C. All solutions were prepared immediately prior to use.

Methotrexate was dissolved in sterile saline and the pH adjusted with 10M NaOH to approximately 8.5. Final concentration was 20 mg/ml. It was administered intraperitoneally, as a single injection, at a dose of 300 mg/kg.

Leucovorin was dissolved in sterile water to a concentration of 10 mg/ml. It was administered intraperitoneally, as a single injection, at a dose of 100 mg/kg.

Bacteria

Coagulase positive Staphylococcus aureus, cultured from a human specimen on chocolate agar, were obtained from the

Bacteriology laboratory of Yale-New Haven Hospital. A single colony was subcultured on sheep blood agar, and all innocula were obtained from this resultant pure culture. Cultures for innoculation were prepared in thioglycollate broth and incubated for 20-24 hours before use. Doses of bacteria in innocula were checked by backplating.

Serum Methotrexate Levels

Certain animals were selected for determination of serum MTX activity (see below). Blood was obtained by random cardiac puncture with a 19-gauge needle, transferred to a red-topped Vacutainer^{R} tube, allowed to clot, and then centrifuged at approximately 2000 rpm for 10 minutes. The serum was aspirated with a Pasteur pipette and then frozen for assay at a later time. Methotrexate activity was determined using the dihydofolate reductase method.[40]

Anesthesia

Animals were anesthetized with Pentosol^{R} (sodium pentobarbital, 6.5%) I.P., approximately 50 mg/kg. An anesthesia death was considered to be a death occurring within two hours of I.P. injection.

Wounding

On the appropriate days after leucovorin administration (see below) groups of rats were anesthetized, their backs were shaved, and they were strapped prone to dissection boards.

The dorsum of each animal was prepped with Betadine^{R} solution.

A sterile #10 scalpel was used to make a 2 cm longitudinal incision. The incision was made to the right of the midline, in a cephalad-caudad direction, from 4 cm to 2 cm above an imaginary line joining the iliac crests. The incision was carried through the skin and panniculus carnosus and into the paraspinous musculature. Bleeding was controlled by pressure with gauze.

Wounds were then innoculated with either 0.1 ml sterile saline or 0.1 ml thioglycollate broth calculated to contain 10^{5} S. aureus (see below). The skin and panniculus were then closed in one layer with three interrupted sutures of 5-0 Ethicon^{R} black, monofilament nylon on an FS-2 needle, and instrument tied with four knots. The wound in the musculature was not sutured. In every group, animals designated to receive saline innocula were wounded before those animals designated to receive innocula of S. aureus. Following wounding, rats were returned to their cages. Bacteria-innoculated animals were isolated from saline-innoculated animals, and MTX-injected animals were caged separately from non-MTX-injected animals. No dressings were applied.

Biopsy

All animals were biopsied four days after wounding. Animals that received saline innocula were biopsied before those that received S. aureus. The rats were anesthetized,

but they were neither shaved nor prepped. Using a #10 scalpel, an incision was made at least 2 cm lateral to the original wound. Subpannicular dissection was carried out to the previous incision in the paraspinous musculature. Under aseptic conditions, a portion of fascia overlying this incision, approximately 0.8 cm x 0.8 cm, was excised.

Each specimen was placed on a separate piece of powder paper (glassine). Then it was flamed, weighed, and ground in a sterile tube with a sterile pestle, after being diluted 1 to 10 with thioglycollate broth. Growth of a single organism from 0.1 ml of the contents of the tube would be equivalent to 10^2 organisms per gram of specimen. Serial dilutions were done in thioglycollate broth to produce blood agar plates which could be read for bacterial counts of 10^2, 10^4, 10^6, and 10^8 organisms per gram. Plates were read after 24 hours of incubation at 37°C.

White Blood Count

After biopsy, each animal was laid supine, the thorax cut open with a scissors, and at least 1 ml of blood was aspirated from the heart under direct visualization. The blood was transferred to purple-topped Vacutainer^R tubes (containing EDTA) and sent to the Hematology laboratory of Yale-New Haven Hospital for automated white blood cell count.

Experimental Procedure

One hundred eight rats were injected with MTX. Nine of

these rats were set aside to observe the lethality of the dose. An additional 9 were sacrificed (in groups of 3) at ½, 4, and 8 hours following injection, and their sera assayed for MTX activity. The remaining 90 rats were injected with leucovorin 24 hours after MTX injection.

Twenty rats were injected with sterile saline I.P. in lieu of MTX and leucovorin, in comparable volumes by weight.

Methotrexate- and non-MTX-injected rats were caged separately. Animals receiving MTX and leucovorin were isolated from those receiving MTX alone.

Rats were divided into 5 groups (Groups I-V). Groups were wounded 1, 3, 7, 14, and 28 days, respectively, following leucovorin (or sham leucovorin) injection. Each Group consisted of 22 rats: 18 MTX- and 4 non-MTX-injected animals. Wound innoculation in each group was carried out as follows:

# of rats	I.P. injection	Innoculum
15	MTX	S. aureus
3	MTX	Saline
2	Saline	S. aureus
2	Saline	Saline

Wound biopsy, for quantitative bacterial count, and white blood cell count were performed on all animals four days after wounding.

Statistical Analysis

Analyses were performed of quantitative bacterial counts and white blood cell counts using a two-tailed student's

"t" test of two independent means.[41] Statistical significance of bacterial counts was computed using the logs of these values. A count of $<10^2$ organisms/g was assigned a value of 1.0.

In each Group, the relationship of elevated bacterial counts to elevated WBC's was assessed by calculation of the Pearson product-moment correlation coefficient.[41] Again, logs of the quantitative counts were used in these determinations.

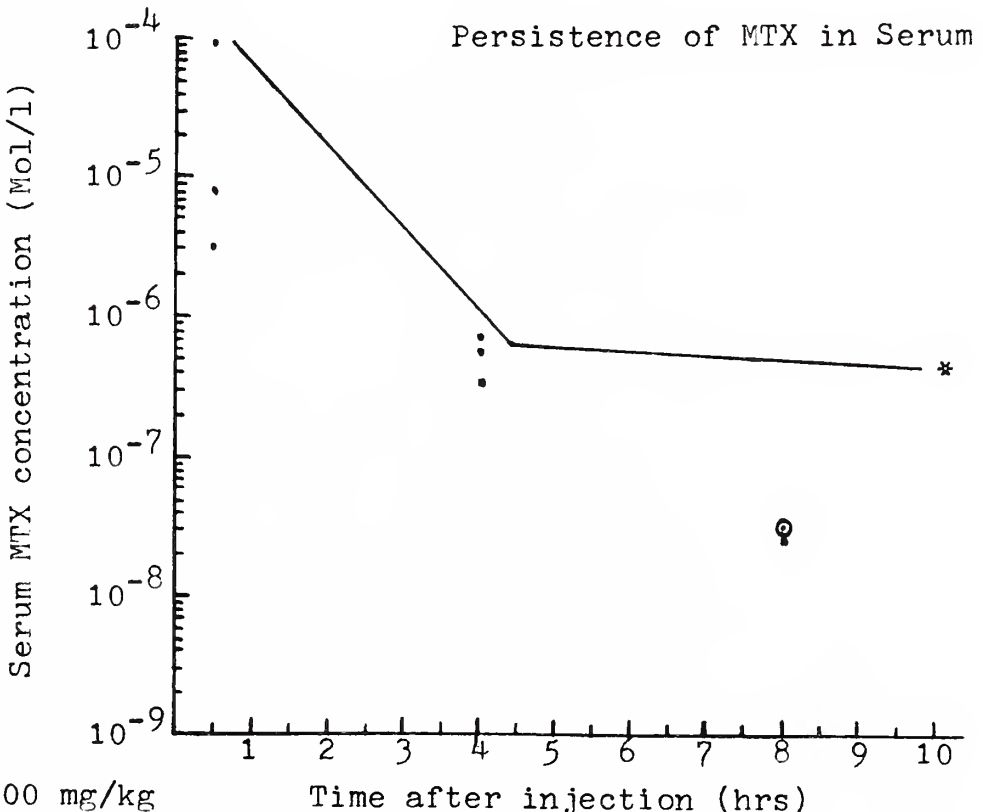
Identification of Bacteria

S. aureus was distinguished by medium-sized, whitish colonies surrounded by zones of beta-hemolysis. Two forms of contaminant were encountered. One was typified by small, greyish-white colonies, without hemolysis, resembling S. epidermidis. These were seen occasionally, though never in great numbers. The other contaminant was typified by large, irregular, greyish colonies, resembling several gram negative species. These were seen rarely.

RESULTS

Serum Methotrexate Levels

Serum MTX levels at ½, 4, and 8 hours after injection are recorded in Table 1. When these values are plotted (Figure 1), they do not describe the complex plasma persistence curve seen by Borsa *et al.* in mice.[42] This may be due either to the initial erratic absorption from the peritoneum, to a difference in metabolism and excretion between the species, or to the small number of rats used here.



*MTX 500 mg/kg
(modified from Borsa *et al.*42)

FIGURE 1

METHOTREXATE LEVELS

Time	Rat #	Serum Concentration
½ hr	106	3.17×10^{-6} M
	107	8.47×10^{-6} M
	108	9.17×10^{-5} M
4 hrs	1	3.53×10^{-7} M
	2	6.11×10^{-7} M
	3	7.76×10^{-7} M
8 hrs	4	3.17×10^{-8} M
	5	3.17×10^{-8} M
	6	2.59×10^{-8} M

TABLE 1

Toxicity/Lethality

There were 4 deaths out of 9 rats that received MTX without leucovorin (44.4%). Deaths occurred 1, 3, and 7 (2) days following MTX administration. Toxic manifestations included weakness, lethargy, and disheveled appearance. Blood was also noted in the feces. Animals that survived demonstrated these signs to a lesser extent, if at all.

Two deaths occurred in rats that received MTX and leucovorin (#'s 26 and 40). These deaths occurred at 8 and 6 days, respectively, following MTX injection. Rat #40 had undergone no procedures (i.e. wounding); it is presumed that MTX toxicity was the cause of death. Rat #26 had been wounded and innoculated with S. aureus 3-4 days before its death. While it is altogether possible that it may have succumbed to MTX, death secondary to sepsis cannot be ruled out.

Susceptibility of rats to fatal intoxication from single doses is known to be variable.[43] This may reflect differences in absorption, state of hydration, and metabolism.

Anesthesia Deaths

There were 2 anesthetic deaths.

The terms to be used in this report with regard to the 4 groups of rats are:

a) MTX-Staph: rats injected with MTX and leucovorin; wounds innoculated with S. aureus

b) MTX-Saline: rats injected with MTX and leucovorin; wounds innoculated with saline

c) Saline-Staph: rats injected with saline; wounds innoculated with S. aureus

d) Saline-Saline: rats injected with saline; wounds innoculated with saline

All raw data are presented in the appendix, Tables A through E.

Quantitative Bacterial Counts

One hundred two rats were wounded. One rat, #26, died 3-4 days after wounding and innoculation with S. aureus. Its death was due either to MTX toxicity or to septicemia. There were 4 dehiscences. These all occurred in MTX-Staph rats. One hundred one biopsies were performed. No gross pus was evident in any wound. Seventeen wounds grew out $>10^5$ organisms

per gram of tissue. All bacterial counts of $>10^5$ organisms/g were from MTX-Staph rats.

The distribution of quantitative counts with respect to time of wounding following MTX-leucovorin administration is recorded in Table 2 and displayed graphically in Figure 2. Twenty-seven percent of the MTX-Staph rats had bacterial counts of $>10^5$ organisms/g. Sixty-nine percent of counts were $<10^5$, 53% were $<10^4$, and 23% were $<10^3$. In contrast, 100% of the wounds of control rats (MTX-Saline, Saline-Staph, and Saline-Saline) had quantitative counts of $<10^5$ organisms/g, 86% of the wounds had counts which were $<10^4$, and 66% were $<10^3$.

In Group I, 33.3% (5/15) of the MTX-Staph rats grew out $>10^5$ organisms/g. Twenty percent (3/15) of wounds dehisced in the MTX-Staph group; two of these subsequently grew out $>10^5$ organisms/g, and one grew out $<10^5$. Analysis of the logs of the quantitative counts of the MTX-Staph rats showed them to be significantly higher than both those of the Group I controls ($p<0.05$) and those of the ten Saline-Staph rats ($p<0.001$). Although the bacterial counts of the Group I MTX-Saline rats were all $<10^5$, they were still significantly higher than those of the ten Saline-Staph rats ($p<0.01$) and also those of the ten Saline-Saline rats ($p<0.02$).

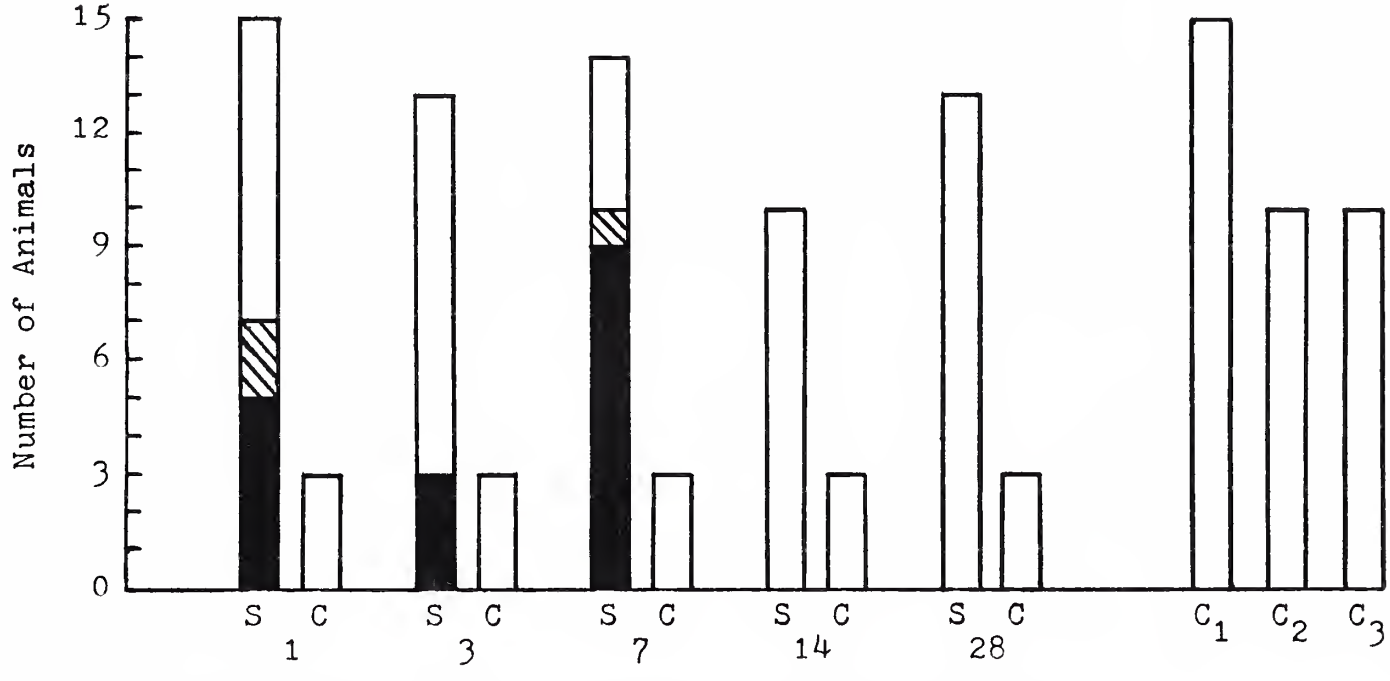
In Group II, 21.4% (3/14) of the MTX-Staph rats grew out $>10^5$ organisms/g. There was one dehiscence; this wound grew out $>10^5$ organisms/g. The quantitative counts of the MTX-Staph rats were significantly higher than those of the

DISTRIBUTION OF QUANTITATIVE BACTERIAL COUNTS

Bacterial Count (organisms/g)	Group I (Day 1)		Group II (Day 3)		Group III (Day 7)		Group IV (Day 14)		Group V (Day 28)		Controls		
	MTX-Staph	MTX-Saline	MTX-Staph	MTX-Saline	MTX-Staph	MTX-Saline	MTX-Staph	MTX-Saline	MTX-Staph	MTX-Saline	C_1 MTX-Saline (all groups)	C_2 Saline-Staph	C_3 Saline-Saline
$1\text{-}9\times10^{8}$					1								
$1\text{-}9\times10^{7}$	1				1								
$1\text{-}9\times10^{6}$	1		2		3								
$2\text{-}9\times10^{5}$	3		1		4								
10^{5}	2				1								
$1\text{-}9\times10^{4}$	4	3	3		3						3	1	1
$1\text{-}9\times10^{3}$	4		3	1	1		8	1	3		2	2	3
$1\text{-}9\times10^{2}$			2	2		3	1	1	8		6	6	5
10^{2}			2					1	2	3	4	1	1
Totals	15		13		14		9		13		15	10	10

TABLE 2

DISTRIBUTION OF QUANTITATIVE BACTERIAL COUNTS



□ $<10^5$

▨ 10^5 organisms/g

■ $>10^5$

S = MTX-Staph rats

C = MTX-Saline rats

C_1 = MTX-Saline rats, all groups

C_2 = Saline-Staph rats

C_3 = Saline-Saline rats

FIGURE 2

Group II controls ($p < 0.05$), but there was no significant difference compared to the ten Saline-Staph rats.

In Group III, 64.3% (9/14) of MTX-Staph rats grew out $>10^5$ S. aureus/g. At the time of biopsy, the wounds of this experimental group demonstrated granulomata. These were not found in any of the controls. Bacterial counts of the MTX-Staph rats were significantly higher than both those of the Group's controls ($p < 0.001$) and those of the ten Saline-Staph rats ($p < 0.001$).

Although wound innocula were calculated to be 10^5 S. aureus, it was found, on backplating, that rats in Group IV received only 10^4 bacteria in their wounds. None of the rats in this Group grew out $>10^5$ S. aureus. In fact, all the wounds cleared the bacteria to $6x10^3$ or less. No significant elevation of bacterial counts was found.

All quantitative counts in Group V were reduced to $3x10^3$ or less per gram. Although counts of this MTX-Staph group were significantly higher than those of its simultaneous controls ($p < 0.05$), there was no statistical difference between these MTX-Staph rats and the ten Saline-Staph rats.

There was no statistically significant difference among the three control groups (MTX-Saline, 15 rats; Saline-Staph, 10 rats; Saline-Saline, 10 rats) with respect to quantitative bacterial count.

White Blood Cell Count

Although the mean white blood count of the MTX-Staph

rats in Group I was less than the mean of the Saline-Staph rats, the difference was not significant. There was, however, an inverse correlation between the quantitative counts and the WBC's; that is, higher bacterial counts correlated with lower white blood counts ($p < 0.05$).

In Group II, WBC's of the MTX-Staph group did not differ significantly from those of any control group.

White cell counts of the MTX-Staph rats in Group III were generally elevated and were significantly higher than those of the Group's simultaneous controls ($p < 0.001$), as well as those of the Saline-Staph group ($p < 0.001$). There was a direct correlation between high quantitative counts and high white blood cell counts for the 21 rats of Group III ($p < 0.001$), but there was no significant correlation between bacterial counts and white cell counts of the 14 MTX-Staph rats.

In Group IV, five MTX-injected rats were not wounded, in order to serve as additional controls for WBC's. The MTX-Staph group had significantly higher white counts than these controls ($p < 0.05$), although there was no significant difference when compared to these rats plus the 3 MTX-Saline control rats. There was no significant difference between the white cell counts of the MTX-Staph rats and those of all Group IV control rats, nor was there any difference in comparison to the Saline-Staph group.

White counts of the Group V MTX-Staph rats were not significantly elevated, but a direct correlation was again seen

between quantitative counts and white blood cell counts for both the MTX-Staph rats ($p < 0.02$) and the entire Group ($p < 0.02$).

There was no significant difference among the MTX-Saline group, the Saline-Staph group, and the Saline-Saline group with respect to white blood cell count.

DISCUSSION

The presence of more than 10^5 bacteria per gram of tissue has been shown to be associated with clinical infection and wound breakdown.[44] Therefore, the term "wound infection" shall henceforth refer to the case where biopsy of a wound contains more than 10^5 organisms per gram of tissue.

In this experiment, then, 26% of rats given high-dose MTX and leucovorin, and subsequently wounded and innoculated with subinfective doses of S. aureus, developed wound infections. All infections occurred in rats wounded within one week following leucovorin administration (or, eight days after MTX), a 40% wound infection rate for MTX-Staph rats during that period. All dehiscences occurred in Groups I and II, which was a rate of 14% of the wounds made within four days of injection of MTX. It is likely that the one rat that had a count of $<10^5$ bacteria had originally developed a wound infection, causing this dehiscence. Rats in the MTX-Saline, Saline-Staph, and Saline-Saline control groups had no infections, and there were no dehiscences.

This experiment clearly shows that MTX has a deleterious effect on the host's ability to tolerate wound contamination. In order to understand exactly how MTX impairs this tolerance, an understanding of the host's natural defense mechanisms is essential.

Normally, the initial barrier to infection is the epithelial surface with which bacteria come into contact. The dryness of the skin, its lysozyme content, and the presence of bactericidal fatty acids in sebaceous secretions all afford protection from bacterial invasion. In the respiratory tract, cilia and mucus remove particulate matter.[44] Polymorphonuclear leukocytes abound in the mucous membrane of the oropharynx, and IgA antibodies in nasal secretions and saliva agglutinate bacteria for more effective phagocytosis.[19] Acidity plays an important role in preventing bacteria from establishing themselves in the alimentary and genito-urinary tracts.[44]

Once the epithelium has been incised, by a scalpel, for example, the systemic defense mechanisms are evoked. These are the processes of inflammation, humoral immunity, and cell-mediated immunity. They attempt to contain and resolve the infectious process.

Acute inflammation is a non-specific response to tissue injury. It begins with constriction of venular sphincters, mediated by certain vasoactive amines, notably histamine and bradykinin. This eventually produces increased vascular permeability and the leakage of protein-rich fluid into the tissues. Blood leukocytes adhere to the endothelium of the venules and capillaries and then emigrate via the widened interendothelial junctions.[45] Chemotaxis is effected by by-products of the complement system, especially C5 fragments, and by clumps of bacteria.[46] In the early stages, infiltration is predominantly by neutrophils, reflecting their high

percentage in the peripheral blood. However, because of the short half-life of neutrophils, their numbers in the inflammatory exudate are gradually replaced by mononuclear cells, which become tissue macrophages.[44]

Neutrophils phagocytize the bacteria. They are aided in this process by specific antibody molecules and complement. These plasma proteins act as opsonins with most bacteria, i.e. they coat the organisms and promote effective phagocytosis. Leukocytes have surface receptors for antibody (Fc receptors) and complement (C3 receptors). Bacteria are destroyed in intracellular vaculoes by lysozyme, hydrogen peroxide, and myeloperoxidase. Macrophages are also effective against the microbes, although they may utilize different enzymes from the neutrophils.[44, 46]

Even though destruction of bacteria occurs primarily at the site of innoculation, a number of organisms are drained by the lymphatics to regional nodes. Here cortical dendritic macrophages partially degrade the bacteria and couple them to preformed RNA. The RNA is brought into contact with small lymphocytes, which undergo a morphologic transformation to large lymphoblasts. A wave of proliferation starts approximately 24-48 hours after introduction of the bacteria and lasts four to 14 days. Proliferation of large lymphoblasts yields large numbers of four different classes of cells. These are: 1) committed large lymphoblasts, 2) plasma cells, 3) differentiated effector small lymphocytes, and 4) memory small lymphocytes.

Humoral immunity, the immunity conferred by antibody, originates with the large lymphoblasts and plasma cells. Large lymphoblasts appear at the site of specific antigen localization, in this case a bacterial innoculum, from four to 10 days after immunization. They crawl between endothelial cells and produce antibody locally. Plasma cells remain in the node and produce most of the circulating antibody. Two major classes of circulating antibody are produced. IgM (19S) production peaks at one to two weeks and generally subsides by 28 days. IgG (7S) production supplants that of IgM and may continue for months or years.[30] Antibody may combine with complement to directly cause lysis of certain strains of gram negative bacteria, but, as noted above, with most bacteria they act as opsonins.[46]

Effector small lymphocytes are responsible for cell-mediated immunity. They localize at an antigen site, undergo lymphoblastoid transformation, and produce several factors that attract macrophages to and localize them at the antigen site. They also recruit non-immune lymphocytes and are directly cytotoxic to foreign cells.[30] Cell-mediated immunity does not play an important primary role in defense against bacterial invasion (M. tuberculosis and M. leprae are two of the exceptions). However, the ability of the body to mount a delayed hypersensitivity reaction correlates with morbidity and mortality from sepsis. A normal cutaneous response is associated with a low incidence of morbidity from infection, partial anergy with increased morbidity and mortality, and

total anergy is associated with a high incidence of mortality from infection.[47]

Memory small lymphocytes recirculate through the body, providing immunologic surveillance. On reexposure to antigen they undergo lymphoblastoid transformation and mount a "secondary immune response."[30]

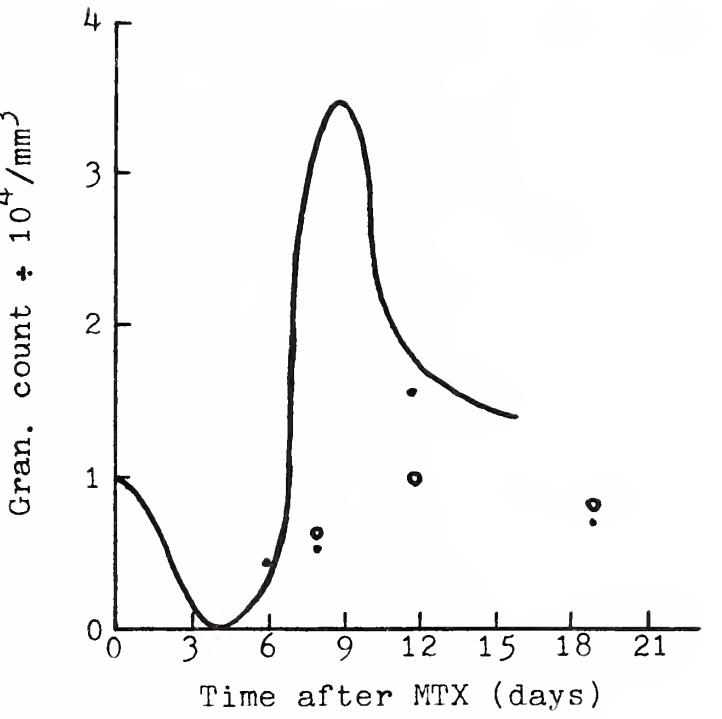
Effect of MTX on the Local Inflammatory Response

That MTX produces a decrease in the peripheral WBC is well known. Rats injected with 50 mg/kg of MTX show a progressive decrease in the number of circulating granulocytes and lymphocytes. The granulocytes are affected to a greater extent, producing a relative lymphocytosis.[43] Condit[48] demonstrated a biphasic response in humans. After I.V. bolus injection of six to 16 mg/kg, total leukocyte counts reached minimum values in four to seven days and recovered to normal levels in seven to 13 days. In 75% of cases, recovery was followed by a second depression of white counts to new minimum values 12-21 days after MTX injection. These values were often lower than during the first depression. Final recovery took anywhere from 15-29 days. These variations essentially reflected changes in the number of circulating neutrophils; the absolute number of lymphocytes varied very little. Maximum leukopenia in the second week after MTX therapy was also noted by Hersh et al.[49, 50] Shortly after treatment, the circulating neutrophils are hypersegmented, like those seen in megaloblastic anemia. These may take as long as one month

to disappear.[51]

Depression of the peripheral white blood count merely reflects concomitant changes in the bone marrow. The number of proliferative cells decreases with increasing doses of MTX, until a plateau is achieved. This plateau represents between 30% and 60% of normal numbers of myeloid precursors.[52, 53] Repeated doses of MTX (every four hours) are able to reduce the number of proliferative cells even further.[54] Granulocyte precursors are predominantly affected. As the lifespan of the granulocyte in the circulation is much shorter than that of the lymphocyte, many more cells in the granulocyte precursor pool undergo DNA replication over a given time period, hence their greater susceptibility to MTX and the relative and absolute neutropenia observed in the peripheral blood.[51]

The ability of the MTX-depressed marrow to mobilize granulocytes in response to infection was studied by Constable and Blackett.[55] Rats were treated with MTX 5 mg/kg. At various intervals afterward, 60 μg of Salmonella typhosa endotoxin were injected I.V., and eight hours later the number of granulocytes in the peripheral blood was examined. Endotoxin response reached a minimum at four days after MTX, with recovery to control levels by seven days. At nine days there was an overshoot to three times the control value, after which the response decreased to normal values at about 16 days. In this experiment, a similar phenomenon was observed with respect to white blood cell counts of the MTX-



• - mean WBC, MTX-Staph rats
o - mean WBC, MTX-Saline rats
— - 8-hour response to S. typhosa endotoxin

FIGURE 3
(modified from Constable and Blackett[55])

Staph rats. Group I rats, biopsied 6 days after MTX injection, had a mean WBC of 4131. By 8 days, white counts averaged 5385. However, Group III MTX-Staph rats, biopsied on day 12 after MTX, had a mean WBC of 15,827. Nineteen days after MTX, average WBC's had returned to 7700 (Group IV). These data are plotted in Figure 3 alongside Constable's response curve and white counts of the respective MTX-Saline controls in the present experiment. (Note: WBC's in the present experiment were drawn four days after bacterial innoculation, as compared to eight hours in Constable's study.) The num-

ber of mobilized granulocytes reflects the size of the pool of mature neutrophilic cells in the bone marrow. The nadir at four days corresponds to the time myeloblasts take to mature into segmented neutrophils; thus, the myeloblast is probably the granulocyte precursor cell most sensitive to MTX.[55]

The neutrophil phase of the inflammatory response is diminished only when the number of granulocytes in the peripheral blood is below 1000/mm^3.[30] The lowest white blood cell count of any MTX-Staph rat was 1500/mm^3, and only two of the rats that developed wound infections had WBC's less than 2000 cells/mm^3. Without differential counts, however, we do not know the absolute number of neutrophils represented. These two rats probably had less than 1000 granulocytes. Nonetheless, the highest infection rate in any Group occurred in the presence of adequate leukocytosis (Group III). However, leukocytes produced at this time may be functionally defective. MTX is known to be ineffective in compromising the metabolism of normal leukocytes, in contrast to its action on acute lymphatic leukemia cells.[72] Therefore, there must be other factors contributing to the susceptibility to infection.

Hersh *et al.*[56] studied the local inflammatory response in man by means of the skin window technique. Patients received either "intensive" or "intermittent" MTX, and skin windows were placed 24 hours after cessation of therapy. When differential counts of the inflammatory exudate were

performed, they showed a significant decrease in the percent of mononuclear cells compared to controls (see Table J in the appendix). The absolute cellularity at four and eight hours approximated that of controls, but at 24 hours total cellularity was clearly depressed (an increased number of neutrophils notwithstanding). Recovery was substantial by five days. The inhibition of mononuclear cell exudation did not correlate with numbers of circulating cells. Leukopenia and stomatitis developed after the skin window tests and persisted for three weeks. Four patients developed infections, including two pneumonias, one pharyngitis with septicemia, and one pharyngitis with furunculosis (see Table K in the appendix). All these patients had very depressed 24-hour mononuclear cell exudates.

It is clear that the mononuclear cell plays an important role in combatting infection. Impaired mononuclear cell exudation also predisposes to the development of Candida albicans infection, even in the face of normal humoral and cell-mediated immunities.[56] Oral candidiasis is a problem not infrequently seen during MTX therapy.[57]

The mechanism of mononuclear cell exudation is unknown.[46] It is not dependent on the presence of neutrophils.[58] Page[59] showed that the process is dependent upon both RNA and protein synthesis. He hypothesized that a substance produced at the site of tissue injury was required by the lymphocyte to initiate mRNA and protein synthesis before the lymphocyte could migrate into the extravascular compartment.

MTX has been shown to significantly inhibit the actions of several vascular permeability factors.[60] Since these proteins are not strictly "chemotactic",[46] they may be the substances of Page's theory. It seems unlikely that the moderate effect of MTX on protein synthesis[61] is responsible for such great inhibition.

Methotrexate, then, is a potent inhibitor of the inflammatory response. It depresses the bone marrow, producing a decrease in circulating granulocytes, and it delays mononuclear cell exudation by several days. In addition, MTX is able to directly inhibit the action of certain factors of vascular permeability. However, it is not known whether leukocyte function is impaired.

Effect of MTX on Humoral Immunity

The local inflammatory response comprises the first line of defense against invading bacteria. A deleterious effect of MTX on humoral immunity would mean the loss of the opsonization function of both local and circulating antibody. The ability of MTX to inhibit cell proliferation would, in theory, make it an effective immunosuppressive agent.

The first investigation of the effect of MTX on humoral immunity was carried out in 1952 by Malmgren *et al*.[62] Mice were injected with sheep red cells intraperitoneally and then treated with a five day course of MTX. Antibody levels, measured by hemolysin titers, decreased with increasing dosage. There was complete absence of antibody effect after

a dose of 1.5 mg/kg/day s.c. There was no inhibition of preformed antibody, either in vitro or in vivo. This suggests that MTX acts at the site of antibody production.

Berenbaum[63] studied the effect of a single injection of MTX on the antibody response of the mouse to T.A.B. (Typhoid-paratyphoid A and B) vaccine. Significant depression of antibody levels occurred when MTX was injected one to two days after vaccination. This would correlate with the wave of proliferation of large lymphoblasts in the node. Antibody levels remained depressed for several weeks afterward. There was no effect if MTX was injected before the vaccination, and there was also no effect on established antibody levels. Berenbaum later expanded upon this model to examine the protective effects of leucovorin.[61] He found that administration of leucovorin between one hour before and one hour after MTX injection prevented the deleterious effect of MTX on antibody production. Antibody levels were partially depressed if leucovorin was delayed for two hours, but a delay of six to eight hours afforded no protection.

Santos and Owens[64] found that mean peak antibody titers were significantly depressed, except when the sheep red blood cells were injected either five days or more before the start of MTX or two days or more after the completion of a five day course of MTX. They subsequently demonstrated the correlation of these effects with 19S and 7S antibody production (see Table L in the appendix).[65] Otterness and Chang[66] demonstrated in mice that MTX injected as a single dose at

the time of immunization was inactive in suppressing antibody synthesis.

The immunosuppressive effect of MTX has also been studied in humans. Hersh et al.[49] exposed patients on "intensive" MTX therapy (daily for five days) and "intermittent" MTX therapy (every fourth day) to Vi antigen, tularemia vaccine, and pneumococcus Type III polysaccharide. The antigen was administered during their courses of therapy, and 71% of patients on intensive MTX therapy and 50% of patients on intermittent therapy showed complete suppression of antibody response. In a follow-up study, they demonstrated the return of normal antibody response when the antigen was administered three days after the completion of MTX therapy.[50] Mitchell et al.[67] studied the response of a series of patients receiving intermittent high-dose MTX and leucovorin rescue to E. coli Vi antigen and tetanus toxoid. Antigens were administered immediately prior to the start of therapy, and as long as infusions were continued, there was complete suppression of all antibody synthesis. After therapy, 55% of patients recovered antibody synthesis within 20 days, which suggests that MTX does not interfere with the initial recognition of antigen by lymphoid cells.

Methotrexate, therefore, is a potent inhibitor of antibody production, but the critical element is the timing of administration of the drug relative to the introduction of an antigen. Maximum antibody suppression is seen when MTX is injected two days after the antigenic stimulus. When the two

events occur simultaneously, MTX has no effect when given as a single injection, but a continuous course will suppress antibody production. In the present study, MTX was injected at least 48 hours before any antigenic stimulus (S. aureus). The results of previous experiments would indicate that there should be no effect on the production of antibody. However, none of the aforementioned studies administered as high a dose of MTX prior to immunization as in the present experiment. Whether or not the dose used in this experiment would suppress antibody production remains still in question.

Effect of MTX on Cell-Mediated Immunity

The effects of MTX on cell-mediated immunity are similar to its effects on humoral immunity. Otterness and Chang[66] demonstrated the inhibition of a primary cellular immune response when antigen was administered during a course of MTX therapy. As a single dose at the time of immunization, though, MTX was inactive. Medzihradsky et al.[68] found MTX to be most effective in suppressing both complement-dependent and complement-independent cellular immunity when administered two days after the antigenic stimulus. Complement-independent cellular cytotoxicity, the kind associated with delayed hypersensitivity, was sensitive to the administration of MTX four days before immunization. However, the appearance of delayed hypersensitivity has been shown by Hersh et al.[49] and Mitchell et al.[67] to be unaffected by MTX, both during and after a course of the drug.

Methotrexate, therefore, is able to inhibit all three aspects of the systemic host defenses. It appears that only the effects on the inflammatory response are of consequence to the present study because of the relative timing of MTX administration and bacterial innoculation.

In addition to the inhibitory effects on the host defense mechanisms, MTX may promote wound infection by otherwise delaying wound healing. For example, bone marrow depression may lead to thrombocytopenia and ineffective hemostasis. Extravascular blood clots and hematomas predispose to infection.[69] Clinically, though, thrombocytopenia has not been a problem.[48, 51]

While bacteria may be directly introduced into a wound, erosion of the oral mucosa and gut epithelium secondary to MTX would expose the tissues to ambient flora, leading to bacteremia and seeding of the operative wound.[44] This may account for the gram negative "contaminants" encountered in wound biopsies.

In the "proliferative phase" of wound healing, there is rapid division of fibroblasts, and epithelial and endothelial cells. MTX is known to inhibit rapidly dividing cell populations. However, by analogy to the immune system, preoperative MTX probably does not affect these cell lines. The effect of MTX on the proliferative phase is most likely due to its effect on the inflammatory phase. Since the proliferative phase begins when the inflammatory phase ends,[70] and since MTX is so effective at inhibiting and prolonging

the inflammatory phase, then the effect of MTX on the proliferative phase is to delay its onset. This may be responsible for the observed experimental effects of MTX on wound healing, discussed above.

The role of the neutrophil in wound healing, aside from defense, is controversial. It has been proposed that neutrophils generate electronically excited singlet molecular oxygen to aid in proline hydroxylation.[71] If this is so, then MTX-induced neutropenia and any functional limitations imposed on neutrophils by MTX might delay wound healing.

Finally, MTX has associated hepatic and renal toxicities. Both uremia and hepatic disease lead to depressed wound healing and an increased risk of infection.[69]

At this point, some of the experimental results become more explicable. For instance, the elevated bacterial counts of the MTX-Saline rats in Group I were the result of the rats' decreased tolerance to their own skin flora. Two days after MTX injection white counts were probably dropping (by day six, one was still 3500; unfortunately, the other two samples clotted). Perhaps the number of granulocytes was less than $1000/mm^3$. A severely inhibited inflammatory response would allow the 10^2-10^3 bacteria per gram that inhabit the surface of the skin to flourish, if only moderately, but to levels significantly above normal.

Another interesting phenomenon is the incidence of wound separation. Twenty percent of MTX-Staph rats wounded

two days after MTX therapy sustained wound dehiscence. In Group II (four days after MTX), the rate of wound separation was 7%. By seven days (Group III), there were no dehiscences, even in the face of the highest infection rate (64%). All rats that suffered wound separation had white blood cell counts at the time of biopsy less than or equal to 3600 cells per mm^3. The interplay of inflammatory response and wound integrity becomes evident. Having less than an adequate number of granulocytes and a strongly inhibited mononuclear cell exudation, the rats are highly susceptible to establishment of infection. Significant tensile strength in a wound does not appear until well into the proliferative phase.[69] However, as long as the stimulus to inflammation is present and a response not forthcoming, proliferation will not begin. Eventually, the bacteria overwhelm the paltry local defenses, and the wounds separate.

As the rats recover from the neutropenia and the delayed inflammatory response, the incidence of wound infection should decrease. A possible explanation for why it does not may be that the neutrophils released in the "overshoot" (see above), although mature morphologically, are somehow immature functionally. A defect in chemotaxis, binding of complement and antibody, phagocytosis, or intracellular digestion would allow infection to be maintained. After another week, the bone marrow returns to the production of functionally competent neutrophils.

CONCLUSION

The results of this experiment indicate that rats treated with a dose of methotrexate in the LD_{50} range and rescued with leucovorin 24 hours later have a decreased tolerance to controlled contamination of surgical wounds. The fact that none of the uncontaminated wounds became infected demonstrates the importance of bacterial contamination in the pathogenesis of wound complications seen clinically in the postoperative period. In the head and neck cancer patient, saliva is an obvious source of such contamination.

Rats were at increased risk for infection only when they were wounded and innoculated within one week of MTX-leucovorin therapy, which supports the clinical impressions of Kiehn and DesPrez. This suggests that the optimal time interval between MTX therapy and surgery which would achieve normal wound healing and the lowest incidence of wound infection would be at least two weeks.

The experimental design employed herein can be expanded to examine the efficacy of systemic and/or topical antimicrobials for prophylaxis or treatment. Such studies might establish further guidelines for future clinical trials.

Preoperative administration of MTX may prove to be a very effective regimen in the treatment of head and neck

cancer. However, prior to the institution of such combined modality therapy, awareness of any and all attendant risks is essential. It is hoped that this study will contribute to that goal.

GROUP I

Rat #	Quantitative Count	WBC
MTX-Staph		
7	$6x10^4$	6000
8	$5x10^3$	X
9	$6x10^6$ - dehiscence	2100
10	$2x10^5$ - dehiscence	1500
11	$1x10^5$	6300
12	$3x10^5$	2200
13	$5x10^5$	3000
14	$5x10^3$ - dehiscence	3400
15	$5x10^3$	7000
16	$8x10^4$	2800
17	$3x10^4$	1800
18	$5x10^3$	6800
19	$1x10^5$	5100
20	$2x10^4$	5700
21	$5x10^7$	X
MTX-Saline		
82	$5x10^4$	X
83	$4x10^4$	3500
84	$5x10^4$	X
Saline-Staph		
109	$1x10^3$	X
110	$6x10^2$	X
Saline-Saline		
119	$7x10^4$	X
120	$<10^2$	X

X denotes sample clotted

TABLE A

GROUP II

Rat #	Quantitative Count	WBC
MTX-Staph		
22	$1x10^4$	1900
23	$1x10^4$	4300
24	$2x10^3$	4700
25	$7x10^3$	9200
26	Dead by day 8 after MTX	
27	$5x10^5$	6400
28	$1x10^6$	4700
29	$1x10^6$ - dehiscence	3600
30	Contaminated	5900
31	$<10^2$	7100
32	$4x10^3$	4600
33	$7x10^2$	7200
34	$8x10^2$	3600
35	$<10^2$	X
36	$3x10^4$	6800
MTX-Saline		
85	$9x10^2$	5500
86	$1x10^3$	8900
87	$3x10^2$	3400
Saline-Staph		
111	$2x10^2$	X
112	$<10^2$	X
Saline-Saline		
121	$1x10^2$	5000
122	$7x10^2$	5500

X denotes sample clotted

TABLE B

GROUP III

Rat #	Quantitative Count	WBC
MTX-Staph		
37	$2x10^7$	10,300
38	$3x10^5$	15,100
39	$1x10^4$	10,400
40	Dead by day 6 after MTX	
41	$3x10^3$	25,400
42	$5x10^8$	18,900
43	$1x10^6$	13,600
44	$2x10^5$	14,700
45	$6x10^6$	15,200
46	$6x10^4$	X
47	$2x10^4$	8600
48	$1x10^5$	X
49	$2x10^5$	26,300
50	$5x10^6$	15,600
51	$6x10^5$	X
MTX-Saline		
88	$7x10^2$	14,600
89	$8x10^2$	7500
90	$6x10^2$	7400
Saline-Staph		
113	$1x10^3$	4700
114	$2x10^4$	8100
Saline-Saline		
123	$2x10^3$	6000
124	$1x10^3$	5600

X denotes sample clotted

TABLE C

GROUP IV

Rat #	Quantitative Count	WBC
MTX-Staph		
52	$2x10^3$	7800
53	$1x10^3$	7000
54	$1x10^3$	X
55	$6x10^2$	7400
56	$2x10^3$	8000
57	$6x10^3$	8600
58	Contaminated	11,500
59	$1x10^3$	X
60	$1x10^3$	4500
61	$3x10^3$	6800
MTX injection; no wound		
62		5700
63		4700
64		5600
65		X
66		5200
MTX-Saline		
91	$<10^2$	6200
92	$5x10^2$	9300
93	$3x10^3$	8200
Saline-Staph		
115	$8x10^2$	8200
116	$6x10^2$	5800
Saline-Saline		
125	$1x10^2$	7700
126	$2x10^3$	X

X denotes sample clotted

TABLE D

GROUP V

Rat #	Quantitative Count	WBC
MTX-Staph		
67	$1x10^2$	4800
68	$1x10^2$	5200
69	$3x10^3$	X
70	10^2	X
71	$6x10^2$	12,500
72	$6x10^2$	8300
73	$1x10^3$	9100
74	$1x10^3$	9800
75	Anesthesia death	
76	$3x10^2$	8100
77	$5x10^2$	10,900
78	$6x10^2$	6600
79	Anesthesia death	
80	$1x10^2$	6800
81	10^2	X
MTX-Saline		
94	10^2	7100
95	10^2	4100
96	10^2	X
Saline-Staph		
117	$1x10^2$	7300
118	$1x10^2$	9100
Saline-Saline		
127	$2x10^2$	7500
128	$1x10^2$	7900

X denotes sample clotted

TABLE E

Effect of Increasing Doses of MTX on Wound Tensile Strength

(Tensile strength measured at 5 days)

Dose of MTX	Tensile Strength (g)
0.125 mg/kg/day	88
0.25 mg/kg/day	81
0.5 mg/kg/day	63
1.0 mg/kg/day	39
Controls (saline)	114

TABLE F

(adapted from Calnan and Davies[29])

Effect of Pre- and Postoperative MTX on Wound Healing

(Tensile strength measured at 5 days)

Treatment	Tensile Strength (g)
0.05 mg/kg/day for 5 days preoperatively	144
0.05 mg/kg/day for 5 days pre- and 5 days postoperatively	133
0.05 mg/kg/day for 5 days postoperatively	122
Controls (saline)	154

TABLE G

(adapted from Calnan and Davies[29])

Protective Effect of Leucovorin

Treatment	Tensile Strength (g) 3 days	7 days
MTX 0.5 mg/kg/day	13	111
MTX 0.5 mg/kg/day plus leucovorin 2.5 mg/kg/day	46	228
Leucovorin 2.5 mg/kg/day	61	238
Controls (saline)	36	220

TABLE H

(adapted from Calnan and Davies[29])

Effect of MTX on Wound Healing

	Breaking Strength (g) Day 3	Day 7	Day 21
MTX 80 mg/kg	64±4	127±7	495±35
Controls	77±5	131±7	553±24

TABLE I

(modified from Cohen et al.[36])

Percent Mononuclear Cells in the Skin Windows of Control Subjects and Patients on Therapy

Therapy		% Mononuclear Cells in Skin Windows					
		Control			On Therapy		
		4 hr	8 hr	24 hr	4 hr	8 hr	24 hr
Control	median	2.5	32	80.5	----------		
	range	0-8	26-49	72-85	----------		
Intensive MTX	median	4	22	86	0	1	2
(daily x 5)	range	4-10	22-33	79-90	0	1-2	0-4
Intermittent MTX	median	3	22	86	1	14	37
(q4d x 6)	range	0-6	4-33	71-100	0-7	2-33	3-73

TABLE J

(modified from Hersh *et al.*[56])

Clinical Data in Patients Who Developed Infection after On-Therapy Window Showed Suppression of the Mononuclear Response

Therapy	24-Hour Mononuclear Response	WBC Count on Day of Skin Test	WBC Count on Day of Infection	Type of Infection	Day of Onset after Skin Test
Intensive MTX	2%	6000	3900	Acute gp α,β Strep. pharyngitis and septicemia	7
Intensive MTX	4%	6700	3600	Acute pharyngitis, generalized furunculoses	4
Intermittent MTX	3%	4600	6800	Primary atypical pneumonia (Mycoplasma)	17
Intermittent MTX	9%	6500	17,000	Primary atypical pneumonia (Mycoplasma)	42

TABLE K

(modified from Hersh et al.[56])

Effect of MTX on 19S and 7S Antibody Production in the Rat

Beginning of MTX Administration*	19S	7S
Two days after immunization**	Decreased, but prolonged	Absent
Five days after immunization	Prolonged	Decreased, with delayed recovery
Seven days before immunization	Prolonged	No effect

*MTX 0.75 mg/kg I.P. qd x 5
**1.0 ml of 10% sheep erythrocytes I.V.

TABLE L

(adapted from Santos and Owens[65])

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