Methotrexate potentiates bradykinin-induced increase in macromolecular efflux from the hamster oral mucosa

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Gao, Xiao-Pei, and Israel Rubinstein. Methotrexate potentiates bradykinin-induced increase in macromolecular efflux from the hamster oral mucosa. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1254–R1262, 1997.—The purpose of this study was to determine whether methotrexate modulates bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa and whether this response is mediated by the L-arginine/nitric oxide biosynthetic pathway. Using intravital microscopy, we found that suffusion of methotrexate alone onto the hamster cheek pouch had no significant effects on leaky site formation and increase in clearance of fluorescein isothiocyanate-labeled dextran (molecular mass, 70 kDa). However, methotrexate significantly potentiated bradykinin-induced responses (P < 0.05). These effects were associated with significant increases in nitrites concentration and guanosine 3′,5′-cyclic monophosphate-like immunoreactivity in the suffusate and were abrogated by N6-nitro-L-arginine methyl ester (L-NNAME) but not N6-nitro-o-arginine methyl ester (o-NNAME). L-Arginine, but not o-arginine, abolished L-NNAME-induced responses. ZnCl2 and indomethacin had no significant effects on methotrexate-induced responses. Methotrexate had no significant effects on adenosine- and ionomycin-induced increases in macromolecular efflux. Collectively, these data indicate that methotrexate amplifies bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa in a specific, receptor- and L-arginine/nitric oxide biosynthetic pathway-dependent fashion.

Microcirculation; inflammation; plasma exudation; nitric oxide; chemotherapy

METHOTREXATE, A STRUCTURAL analogue of folic acid, is an effective antineoplastic drug (3). It also exhibits potent anti-inflammatory and anti-fibrotic activities and is used to treat nonmalignant conditions, such as asthma, rheumatoid arthritis, and psoriasis (9, 17, 21, 38). Unfortunately, oral mucositis is a relatively common side effect of methotrexate therapy that leads to significant morbidity (2, 10, 18). A characteristic histopathological feature of this process is plasma exudation and interstitial edema (7, 8, 32, 33, 37). For instance, Sklar (30) showed that subcutaneous injection of methotrexate for 4 wk elicits erythema in the oral mucosa of hamsters. However, the mechanisms underlying the genesis of oral mucositis during methotrexate therapy are uncertain (8, 30, 32).

It is well established that bradykinin, a ubiquitous 9-amino acid phlogistic peptide (1), is produced in the oral mucosa during the host inflammatory response to injury (11–13, 15, 22, 39). Bradykinin elicits plasma exudation from the oral mucosa that is mediated, in part, by the L-arginine/nitric oxide (NO) biosynthetic pathway (4, 12, 19, 20, 29, 34). To this end, Mayhan (20) and Gao and Rubinstein (12) showed that N6-monoacyl-L-arginine (L-NMMA) and N6-nitro-L-arginine methyl ester (L-NNAME), two NO synthase inhibitors, but not N6-monomethyl-o-arginine (o-NMMA) and N6-nitro-o-arginine methyl ester (o-NNAME), respectively, attenuate bradykinin-induced increase in macromolecular efflux from the in situ hamster cheek pouch. Whether bradykinin plays a role in the genesis of oral mucositis associated with methotrexate therapy is unknown.

Hence, the purpose of this study was to begin to address this issue by determining whether methotrexate modulates bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa and whether this response is mediated by the L-arginine/NO biosynthetic pathway.

METHODS

Preparation of Animals

Adult male golden Syrian hamsters weighing 129 ± 1 g (n = 76) were anesthetized with pentobarbital sodium (6 mg/100 g body wt ip). A tracheotomy was performed to facilitate spontaneous breathing. A femoral vein was cannulated to inject the intravascular tracer, fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular mass 70 kDa; 40 mg/100 g body wt dissolved in 1.0 ml normal saline and administered over 1 min) and supplemental anesthesia (2–4 mg·100 g body wt−1·h−1). A femoral artery was cannulated to obtain arterial blood samples and to monitor arterial blood pressure, which did not change significantly during the experiments. Body temperature was kept constant (37–38°C) throughout the experiment using a heating pad.

To visualize the microcirculation of the cheek pouch, we used a method previously described in our laboratory (11–14, 27, 28, 34, 39). Briefly, the left cheek pouch was spread gently over a small plastic base plate, and an incision was made in the outer skin to expose the cheek pouch membrane. The avascular connective tissue layer was removed, and a plastic chamber was positioned over the base plate and secured in place by suturing the skin around the upper chamber. This chamber contained the suffusion fluid. This arrangement forms a triple-layered complex: the base plate, the upper chamber, and the cheek pouch membrane exposed between the two plates. After these initial procedures, the hamster was transferred to a heated microscope stage. The chamber was connected to a reservoir containing warmed bicarbonate buffer (37–38°C) composed (in mM) of 131.9 NaCl, 2.95 KCl, 1.48 CaCl2, 0.76 MgCl2, and 11.87 NaHCO3, which allowed continuous suffusion of the cheek pouch. The buffer was bubbled continuously with 95% N2-5% CO2 (pH 7.4). The chamber was also connected via a three-way valve to an infusion pump (Sage Instruments, Boston, MA) that allowed for the constant administration of drugs into the suffusate.

Determination of Clearance of Macromolecules

The cheek pouch microcirculation was visualized with an Olympus microscope (Jacobs Instruments, Shawnee Mission, Kansas). Dextran (molecular mass, 70 kDa) was administered over 1 min, and supplemental anesthesia was given. A tracheotomy was performed to facilitate spontaneous breathing. A femoral vein was cannulated to inject the intravascular tracer, fluorescein isothiocyanate-labeled dextran (FITC-dextran; molecular mass 70 kDa; 40 mg/100 g body wt dissolved in 1.0 ml normal saline and administered over 1 min) and supplemental anesthesia (2–4 mg·100 g body wt−1·h−1). A femoral artery was cannulated to obtain arterial blood samples and to monitor arterial blood pressure, which did not change significantly during the experiments. Body temperature was kept constant (37–38°C) throughout the experiment using a heating pad.

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The concentration of guanosine 3',5'-cyclic monophosphate (cGMP)-like immunoreactivity in the suffusate was determined in duplicate using a commercial cGMP enzyme-linked immunosorbent assay (ELISA) kit (Amersham, Arlington Heights, IL) according to the manufacturer’s specifications. The phosphodiesterase inhibitor 3-isobutyl-1-methylxantine (1.0 mM) was added to samples to avoid possible interference by changes in phosphodiesterase activity during application of test compounds and to facilitate cGMP accumulation. Optical density was read at 450 nm within 30 min at room temperature using a thermoregulated ELISA microplate reader (Molecular Devices). The sensitivity of the assay is 14 pg/ml, and cross-reactivity with adenosine 3',5'-cyclic monophosphate is <0.00008%.

Experimental Protocols

Effects of methotrexate on bradykinin-induced responses. The purpose of these studies was to determine whether methotrexate potentiates bradykinin-induced leaky site formation and increase in clearance of FITC-dextran from the cheek pouch. After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Then, two concentrations of bradykinin (0.25 and 0.5 µM) were suffused onto the cheek pouch in an arbitrary order. Each concentration was suffused for 5 min (11, 12, 39). The number of leaky sites was determined every minute for 7 min and at 5-min intervals for 45 min thereafter. Clearance of FITC-dextran was determined before and every 5 min for 45 min. The time interval between subsequent suffusions of bradykinin was at least 30 min (11, 12, 15, 39). Once suffusion of bradykinin was stopped and the number of leaky sites returned to baseline, methotrexate (5 mg/kg) was suffused for 30 min at a flow rate of 2 ml/min (final concentration in suffusate, 2.3 × 10^-5 M), and suffusion of bradykinin was repeated. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that repeated suffusions of bradykinin (0.25 and 0.5 µM) before and after suffusion of saline (vehicle) for 30 min were associated with reproducible results. In addition, suffusion of saline for the entire duration of the experiment was not associated with visible leaky site formation and increase in clearance of FITC-dextran. The concentrations of bradykinin and methotrexate used in these studies were based on previous studies in our laboratory and reports in the literature (2, 11, 12, 15, 17, 19, 20, 39).

Effects of L-NAME. The purpose of these studies was to determine whether the L-arginine/NO biosynthetic pathway mediates methotrexate potentiation of bradykinin-induced responses. After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Then, two concentrations of bradykinin (0.25 and 0.5 µM) were suffused as outlined above. Once suffusion of bradykinin was stopped and the number of leaky sites returned to baseline, methotrexate (5 mg/kg) was suffused either in the absence or presence of N^G-nitro-L-arginine methyl ester (L-NAME; 100 µM), an NO synthase inhibitor (11, 20, 27), for 30 min followed by suffusion of bradykinin (0.25 µM and 0.5 µM). In some experiments, D-NAME (100 µM) was used rather than L-NAME. In another group of animals, bradykinin (0.5 µM) was suffused for 5 min before and after suffusing L-arginine (1 mM), the substrate for NO synthase (27, 29); L-NAME (100 µM) and methotrexate (5 mg/kg); or D-arginine (1 mM), L-NAME (100 µM), and methotrexate (5 mg/kg) for 30 min. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that suffusion of L-NAME, D-NAME, L-arginine, and D-arginine alone for 30 min was not associated with visible leaky site formation and increase in clear-
brane of FITC-dextran. The concentrations of L-NAME, D-NAME, L-arginine, and D-arginine used in these studies were based on previous studies in our laboratory and reports in the literature (20, 27, 29, 31, 34).

In another series of experiments, the suffusate was collected into prechilled polypropylene test tubes during the last 5 min of 30-min suffusion period of saline (control), methotrexate (5 mg/kg), and L-NAME (100 µM) alone. The suffusate was also collected during 5-min suffusion of bradykinin (0.5 µM) alone, bradykinin (0.5 µM) after suffusion of methotrexate (5 mg/kg) for 30 min, and bradykinin (0.5 µM) after suffusion of methotrexate (5 mg/kg) and L-NAME (100 µM) for 30 min. Samples were immediately snap-frozen in liquid nitrogen and stored at −70°C until used for determination of nitrites concentration and cGMP-like immunoreactivity.

Effects of ZnCl₂. The purpose of these studies was to determine whether methotrexate potentiation of bradykinin-induced increase in macromolecular efflux is partly related to chelation of the zinc moiety in catalytic domains of metalloenzymes, such as angiotensin I-converting enzyme and neutral endopeptidase 24.11, that cleave and inactivate bradykinin in the cheek pouch (5, 16, 26, 36, 39). After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Thereafter, bradykinin (0.5 µM) was suffused for 5 min before and after ZnCl₂ was suffused (100 µM) for 30 min. In another group of animals, bradykinin (0.5 µM) was suffused for 5 min before and after suffusion of methotrexate (5 mg/kg) alone or methotrexate (5 mg/kg) with ZnCl₂ (100 µM) for 30 min. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that suffusion of ZnCl₂ (100 µM) alone for 30 min was not associated with visible leaky site formation and increase in clearance of FITC-dextran. The concentration of ZnCl₂ used in these studies was based on preliminary studies and a previous report in the literature (16).

Effects of indomethacin. The purpose of these studies was to determine whether products released through the cyclooxygenase pathway of arachidonic acid metabolism mediate, in part, the potentiating effects of methotrexate on bradykinin-induced responses. After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Thereafter, bradykinin (0.5 µM) was suffused for 5 min before and after methotrexate was suffused (5 mg/kg) for 30 min. Then, indomethacin (10 mg/kg) was injected intravenously over a 30-min period using an infusion pump (final volume = 1.0 ml), and suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) was repeated. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. The concentration of indomethacin used in these studies was based on previous studies in our laboratory and a report in the literature (11, 12, 25, 28).

Effects of methotrexate on adenosine-induced responses. The purpose of these studies was to determine the specificity of methotrexate potentiation of bradykinin-induced responses by determining its effects on adenosine-induced leaky site formation and increase in clearance of FITC-dextran from the cheek pouch. A dose of 10 mg/kg was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Thereafter, adenosine (0.5 µM) was suffused for 5 min before and after methotrexate (5 mg/kg) was suffused for 30 min (15, 22, 39). The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that repeated suffusions of adenosine (0.5 µM) were associated with reproducible results. The concentration of adenosine used in these studies was based on previous studies in our laboratory and reports in the literature (11, 14, 15, 22, 39).

Effects of methotrexate on ionomycin-induced responses. The purpose of these studies was to determine whether methotrexate potentiation of bradykinin-induced responses through the L-arginine/NO biosynthetic pathway is mediated, in part, by a receptor-dependent mechanism(s) by determining its effects on ionomycin-induced leaky site formation and increase in clearance of FITC-dextran from the cheek pouch. Calcium ionophores, such as A-23187 and ionomycin, elicit endothelium-dependent, receptor-independent production of NO or a related compound(s) in the cheek pouch (11, 19). After bicarbonate buffer was suffused for 30 min (equilibration period), FITC-dextran was injected intravenously, and the number of leaky sites and clearance of FITC-dextran were determined for 30 min. Then, two concentrations of ionomycin (0.1 and 1.0 nM) were suffused for 5 min in an arbitrary order. At least 45 min elapsed between subsequent suffusions of ionomycin (11, 19). Once suffusion of ionomycin was stopped and the number of leaky sites returned to baseline, methotrexate (5 mg/kg) was suffused for 30 min and suffusion of ionomycin (0.1 and 1.0 nM) was repeated. The number of leaky sites and clearance of FITC-dextran were determined during each intervention. In preliminary studies, we determined that repeated suffusions of ionomycin (0.1 and 1.0 nM) were associated with reproducible results. The concentrations of ionomycin used in these studies were based on a previous study in our laboratory and a report in the literature (11, 19).

Drugs. FITC-dextran, methotrexate, bradykinin, L-NAME, D-NAME, L-arginine, D-arginine, ZnCl₂, indomethacin, adenosine, ionomycin, Escherichia coli nitrate reductase, NaNO₃, NaNO₂, and 3-isobutyl-1-methylxanthine were obtained from Sigma Chemical (St. Louis, MO). Indomethacin was dissolved in sodium bicarbonate. All drugs were diluted in saline to the desired concentrations on the day of the experiment.

Data and statistical analyses. When a test compound was suffused over the cheek pouch, we determined the maximal change in the number of leaky sites and clearance of FITC-dextran and used it as the response to that compound. Data are expressed as means ± SE. Because the number of leaky sites and clearance of FITC-dextran returned to baseline between successive suffusions of test compounds, all control (saline) data are expressed as a single value for each experimental condition. Statistical analysis was performed using two-way analysis of variance and the Newman-Kuels test for multiple comparisons. P < 0.05 was considered significant, and n is given as the number of experiments, with each experiment representing a separate animal.

RESULTS

Effects of Methotrexate on Bradykinin-Induced Responses

Suffusion of methotrexate (5 mg/kg) alone for 30 min was not associated with visible leaky site formation and increase in clearance of FITC-dextran (data not shown; n = 6). However, it significantly potentiated bradykinin-induced responses (Fig. 1; each group, n = 6; P < 0.05).
The number of leaky sites increased significantly from $10 \pm 1/0.11 \text{ cm}^2$ during suffusion of bradykinin (0.5 µM) alone to $19 \pm 2/0.11 \text{ cm}^2$ during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 1A; each group, n = 6; P < 0.05). Likewise, clearance of FITC-dextran increased significantly from $26.5 \pm 4.5 \text{ ml/min} \times 10^{-6}$ during suffusion of bradykinin (0.5 µM) alone to $36.7 \pm 6.4 \text{ ml/min} \times 10^{-6}$ during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 1B; each group, n = 6; P < 0.05).

Effects of L-NAME

Suffusion of L-NAME, but not D-NAME (each, 100 µM), abrogated the potentiating effects of methotrexate (5 mg/kg) on bradykinin (0.5 µM; Fig. 2A; each group, n = 4; P < 0.05). Clearance of FITC-dextran also decreased significantly from $39.4 \pm 4.6 \text{ ml/min} \times 10^{-6}$ during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) to $22.3 \pm 4.1 \text{ ml/min} \times 10^{-6}$ during suffusion of L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 2B; each group, n = 4; P < 0.05).

Suffusion of L-arginine, but not D-arginine (each, 1 mM), with L-NAME (100 µM) abolished the effects of L-NAME on methotrexate (5 mg/kg)-induced responses (Fig. 2; each group, n = 4). The number of leaky sites increased significantly from $6 \pm 1/0.11 \text{ cm}^2$ during suffusion of L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM) to $23 \pm 2/0.11 \text{ cm}^2$ during suffusion of L-arginine (1 mM), L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 2A; each group, n = 4; P < 0.05). Clearance of FITC-dextran increased significantly from $12.2 \pm 2.4 \text{ ml/min} \times 10^{-6}$ during suffusion of L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM) to $30.3 \pm 4.0 \text{ ml/min} \times 10^{-6}$ during suffusion of L-arginine (1 mM), L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 2B; each group, n = 4; P < 0.05).

Suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) was associated with a significant increase in $\mu$M; Fig. 2A; each group, n = 4; P < 0.05).
nitrates concentration in the suffusate relative to suffusion of methotrexate and bradykinin alone (Table 1; 12.00 ± 0.32 vs. 7.06 ± 0.19 and 9.35 ± 0.63 µM, respectively; each group, n = 4; P < 0.05). L-NAME (100 µM) abrogated the increase in nitrites concentration in the suffusate during suffusion of methotrexate and bradykinin (Table 1). cGMP-like immunoreactivity in the suffusate also increased significantly during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM) relative to suffusion of methotrexate and bradykinin alone (Table 1; 201.45 ± 3.00 pg/ml vs. 58.95 ± 0.29 and 115.90 ± 2.50 pg/ml, respectively; each group, n = 4; P < 0.05). L-NAME (100 µM) abrogated the increase in cGMP-like immunoreactivity in the suffusate during suffusion of methotrexate and bradykinin (Table 1).

### Effects of ZnCl₂

Suffusion of ZnCl₂ (100 µM) had no significant effects on bradykinin (0.5 µM)-induced leaky site formation and increase in clearance of FITC-dextran (Fig. 3A; each group, n = 4; P > 0.5). ZnCl₂ (100 µM) had also no significant effects on methotrexate (5 mg/kg) potentiation of bradykinin (0.5 µM)-induced responses (Fig. 3B; each group, n = 6; P > 0.5). The number of leaky sites was 24 ± 2/0.11 cm² during suffusion of methotrexate (5 mg/kg) and bradykinin (0.5 µM), and 21 ± 2/0.11 cm² during suffusion of ZnCl₂ (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; Fig. 3B, top; each group, n = 6; P > 0.5). Likewise, clearance of FITC-dextran was 32.6 ± 5.2 ml/min x 10⁻⁶ during suffusion

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**Table 1. Nitrites concentration and cGMP-like immunoreactivity in cheek pouch suffusate**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Nitrites, µM</th>
<th>cGMP, pg/ml</th>
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<tbody>
<tr>
<td>Bicarbonate buffer</td>
<td>0.15 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>0.93 ± 0.09</td>
<td>ND</td>
</tr>
<tr>
<td>Methotrexate (5 mg/kg)</td>
<td>7.06 ± 0.19*</td>
<td>58.95 ± 0.29*</td>
</tr>
<tr>
<td>Bradykinin (0.5 µM)</td>
<td>9.35 ± 0.63*</td>
<td>115.90 ± 2.50†</td>
</tr>
<tr>
<td>Methotrexate and bradykinin</td>
<td>12.00 ± 0.32†</td>
<td>201.45 ± 3.00‡</td>
</tr>
<tr>
<td>L-NAME (100 µM)</td>
<td>4.06 ± 0.40*</td>
<td>ND</td>
</tr>
<tr>
<td>L-NAME, methotrexate, and bradykinin</td>
<td>9.03 ± 0.27*</td>
<td>28.05 ± 0.25§</td>
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</table>

Values are means ± SE; each group, n = 4 experiments. ND, not detected; L-NAME, N⁶-nitro-L-arginine methyl ester. *P < 0.05 in comparison to control; †P < 0.05 in comparison to methotrexate; ‡P < 0.05 in comparison to methotrexate and bradykinin alone; §P < 0.05 in comparison to methotrexate and bradykinin.
of methotrexate (5 mg/kg) and bradykinin (0.5 µM), and 29.1 ± 3.7 ml/min × 10^{-6} during suffusion of ZnCl₂ (100 
µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM; 
Fig. 3, bottom; each group, n = 6; P > 0.5).

Effects of Indomethacin

Indomethacin (10 mg/kg iv) had no significant effects on methotrexate (5 mg/kg) potentiation of bradykinin 
(0.5 µM)-induced responses (Fig. 4; each group, n = 7; 
P > 0.5). The number of leaky sites was 25 ± 3/0.11 cm² 
during suffusion of methotrexate (5 mg/kg) and brady-
kinin (0.5 µM) and was 22 ± 3/0.11 cm² after intrave-
nous infusion of indomethacin (10 mg/kg) and suffusion 
of methotrexate (5 mg/kg) and bradykinin (0.5 µM; Fig. 4A; 
each group, n = 7; P > 0.5). Clearance of FITC-dextran was 
40.1 ± 7.0 ml/min × 10^{-6} during suffusion of methotrexate 
(5 mg/kg) and bradykinin (0.5 µM) and was 31.3 ± 7.0 
ml/min × 10^{-6} after intravenous infusion of indomethacin 
(10 mg/kg) and suffusion of methotrexate (5 mg/kg) and 
bradykinin (0.5 µM; Fig. 4B; each group, n = 7, P > 0.5).

Effects of Methotrexate on 
Adenosine-Induced Responses

Suffusion of methotrexate (5 mg/kg) had no signifi-
cant effects on adenosine (0.5 µM)-induced leaky site for-
mation (Fig. 5; each group, n = 7; P > 0.5). The number of leaky 
sites was 10 ± 3/0.11 cm² during suffusion of adenosine 
(0.5 µM) alone, and 9 ± 2/0.11 cm² during suffusion of methotrexate (5 mg/kg) and adenosine (0.5 µM; Fig. 5A; 
each group, n = 7; P > 0.5). Clearance of FITC-dextran was 
28.3 ± 3.6 ml/min × 10^{-6} during suffusion of adenosine (0.5 µM) alone, and 25.8 ± 5.6 ml/min × 10^{-6} 
during suffusion of methotrexate (5 mg/kg) and adeno-
sine (0.5 µM; Fig. 5B; n = 7; P > 0.5).
Effects of Methotrexate on Ionomycin-Induced Responses

Suffusion of methotrexate (5 mg/kg) had no significant effects on ionomycin (0.1 and 1.0 nM)-induced responses (Fig. 6; each group, n = 6; P > 0.5). The number of leaky sites was 9 ± 2/0.11 cm² during suffusion of ionomycin (1.0 nM) alone and was 6 ± 1/0.11 cm² during suffusion of methotrexate (5 mg/kg) and ionomycin (1.0 nM; Fig. 6A; each group, n = 6; P > 0.5). Clearance of FITC-dextran was 28.6 ± 6.7 ml/min × 10⁻⁶ during suffusion of ionomycin (1.0 nM) alone, and 30.3 ± 6.0 ml/min × 10⁻⁶ during suffusion of methotrexate (5 mg/kg) and ionomycin (1.0 nM; Fig. 6B; each group, n = 6; P > 0.5).

DISCUSSION

The results of this study show that methotrexate, at a concentration used in humans (3, 17), significantly potentiated bradykinin-induced increase in macromolecular efflux from the in situ hamster cheek pouch. These effects were mediated by the L-arginine/NO biosynthetic pathway because they were abrogated by L-NAME, but not d-NAME, and because L-arginine, but not d-arginine, abolished L-NAME-induced responses. Moreover, suffusion of methotrexate and bradykinin was associated with significant increases in nitrites concentration and cGMP-like immunoreactivity in the suffusate that were abrogated by L-NAME. The effects of methotrexate were not related to nonspecific damage to the endothelium because the number of leaky sites and clearance of FITC-dextran returned to baseline once suffusion of methotrexate and bradykinin was stopped.

We also found that methotrexate potentiation of bradykinin-induced responses were specific and receptor dependent because methotrexate had no significant effects on adenosine- and ionomycin-induced increases in macromolecular efflux, and because ZnCl₂ and indo-methacin, at a concentration known to inhibit cyclooxygenase in the cheek pouch (25, 28), had no significant effects on methotrexate-induced responses. On balance, these data suggest that methotrexate potentiation of bradykinin-induced increase in macromolecular efflux from the in situ cheek pouch is mediated by a specific, receptor- and L-arginine/NO biosynthetic pathway-dependent mechanism(s) (19, 27, 29). Further studies are indicated to elucidate the cellular source(s) of NO or a related compound(s) produced in the cheek pouch during suffusion of methotrexate and bradykinin.

The hamster cheek pouch is an established model to investigate mechanisms underlying plasma exudation from the oral mucosa during the host inflammatory response to injury (11–15, 19, 20, 22, 25, 28, 30, 34, 35, 39). To this end, we and others showed that bradykinin is produced in the inflamed oral mucosa and increases macromolecular efflux from postcapillary venules, in part, through the L-arginine/NO biosynthetic pathway (1, 6, 13, 19, 20, 22, 23, 34; and D. R. Springall and I. Rubinstein, unpublished observations). Moreover, Gao et al. (11, 12) showed recently that the edemagenic effects of bradykinin in this organ are potentiated by potent endogenous phlogistic mediators, such as interleukin 1β and a stable analogue of vasoactive intestinal peptide, which by themselves have no significant effects on macromolecular efflux. These potentiating effects were mediated, in part, by the L-arginine/NO biosynthetic pathway. The results of this study support and extend these observations by showing that the L-arginine/NO biosynthetic pathway also mediates methotrexate potentiation of bradykinin-induced increase in macromolecular efflux from the in situ cheek pouch. Whether other antineoplastic drugs amplify the edemagenic effects of bradykinin in the oral mucosa by similar mechanisms remains to be determined (7, 10, 32, 33, 37).

Rubinstein and Mayhan (27) showed that suffusion of L-NAME onto the cheek pouch is associated with significant vasoconstriction. Hence, changes in vasoconstrictor tone and/or venular driving pressure in the cheek pouch might have mediated, in part, methotrexate...
potentiation of bradykinin-induced responses and the attenuating effects of L-NAME. However, this possibility seems unlikely because methotrexate had no significant effects on adenosine- and ionomycin-induced increases in macromolecular efflux. In addition, Tomeo and Durán (35) showed that platelet-activating factor increases macromolecular efflux from the cheek pouch while at the same time eliciting potent vasoconstriction. Finally, Murray et al. (22) showed that bradykinin-induced increase in macromolecular efflux from this organ is not mediated by changes in venular driving pressure. Overall, these data suggest that the effects of methotrexate and L-NAME on bradykinin-induced increase in macromolecular efflux observed in this study could not be attributed to local changes in vasomotor tone or venular driving pressure.

Although suffusion of methotrexate alone was associated with a significant increase in nitrates concentration and cGMP-like immunoreactivity in the suffusate, which was of smaller magnitude relative to that associated with suffusion of bradykinin, it nonetheless had no significant effects on macromolecular efflux. These data are consistent with previous studies in hamsters and rats showing that acute administration of methotrexate is not associated with oral mucositis (10, 30, 32). However, methotrexate potentiated bradykinin-induced increase in macromolecular efflux, and this response was associated with significant increases in nitrates concentration and cGMP-like immunoreactivity in the suffusate, which were greater than those associated with suffusion of each of these compounds alone. The smaller increment in cGMP-like immunoreactivity in the absence of significant changes in macromolecular efflux during suffusion of methotrexate alone suggests that disruption of the barrier function of postcapillary venules in the cheek pouch could be partly related to the magnitude of local cGMP production. Alternatively, nitrates and cGMP-like immunoreactivity produced in the cheek pouch during suffusion of methotrexate alone might be derived from cells not directly involved in regulation of barrier function of postcapillary venules. Clearly, additional studies are warranted to support or refute these hypotheses.

Previous studies showed that inactivation of alcohol dehydrogenase by NO and peroxynitrite is partly related to chelation of the zinc moiety in the catalytic domain of the enzyme (5, 16). In addition, Park and Means (24) showed that sodium nitroprusside, an NO donor, inactivates angiotensin-converting enzyme inhibitor (ACE), a zinc metalloenzyme that hydrolyzes bradykinin very effectively in the cheek pouch (12, 26, 39). Conceivably, methotrexate potentiation of bradykinin-induced increase in macromolecular efflux from this organ could have been related, in part, to chelation of zinc moiety in the catalytic domain of ACE or other metalloenzymes that hydrolyze bradykinin thereby slowing local peptide catabolism and amplifying macromolecular efflux (1, 24, 26, 36, 39). Although we cannot refute this possibility, it nonetheless seems unlikely because suffusion of a relatively high concentration of ZnCl₂ to allow reincorporation of Zn²⁺ had no significant effects on methotrexate potentiation of bradykinin-induced responses.

Perspectives

The specific interaction between methotrexate and bradykinin to amplify macromolecular efflux from the oral mucosa observed in this study suggests that premorbid phenotypic expression of phlogistic mediators in the oral mucosa could play a role in the genesis of oral mucositis during methotrexate therapy.

In summary, we found that methotrexate amplifies bradykinin-induced increase in macromolecular efflux from the in situ oral mucosa in a specific, receptor- and L-arginine/NO biosynthetic pathway-dependent fashion.

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