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 N^6-nitro-L-arginine methyl ester (L-NAME) but not N^6-nitro-o-arginine methyl ester (o-NAME). L-Arginine, but not o-arginine, abolished L-NAME-induced responses. ZnCl_2 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 N^6-mono-

methyl-L-arginine (L-NMMA) and N^6-nitro-L-arginine methyl ester (L-NAME), two NO synthase inhibitors, but not N^6-monomethyl-o-arginine (o-NMMA) and N^6-nitro-o-arginine methyl ester (o-NAME), 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·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 CaCl_2, 0.76 MgCl_2, and 11.87 NaHCO_3, which allowed continuous suffusion of the cheek pouch. The buffer was bubbled continuously with 95% N_2-5% CO_2 (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 (Leica Instruments, Shawnee Mission, KS). A canvas was fixed to the upper surface of the cheek pouch membrane with a cotton thread and a trilaminar filter paper disk was placed on the canvas and covered with a glass plate. The cheek pouch was illuminated with a light source attached to the microscope and the fluorescent image was recorded on a video cassette recorder (VHS). For each animal, the following responses were quantitated: (1) total number of leaky sites, (2) area of leakage of 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 supplemented anesthesia (2–4 mg/100 g body wt·h⁻¹)), and (3) rate of clearance of 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 supplemented anesthesia (2–4 mg/100 g body wt·h⁻¹)).
cGMP Assay

Subtracted from experimental values.

Heights, IL) according to the manufacturer’s specifications.

immunosorbent assay (ELISA) kit (Amersham, Arlington

mined in duplicate using a commercial cGMP enzyme-linked

(cGMP)-like immunoreactivity in the suffusate was deter-

mined in all plasma samples. To quantitate the concentra-

tion of FITC-dextran in plasma and suffusate, a

standard curve for FITC-dextran concentrations versus per-

cent emission was performed on a spectrophotofluorometer

(Photon Technology International, Princeton, NJ). The stan-

dard was FITC-dextran that was prepared on a weight per

volume basis. With the bicarbonate buffer used as back-

ground, a standard curve was generated for each experiment,

and each curve was subjected to linear regression analysis.

The percent emission for unknown samples (plasma and

suffusate) was measured on the spectrophotofluorometer, and

the concentration of FITC-dextran was calculated from the

standard curve. In preliminary experiments, minimal fluores-

cence signal (<2% above background) was detected when

drugs were added to the buffer and when plasma and

suffusate samples were examined before addition of FITC-

dextran. Clearance of FITC-dextran was determined by calcu-

lating the ratio of suffusate (ng/ml) to plasma (mg/ml) concen-

tration of FITC-dextran and multiplying this ratio by the

suffusate flow rate (2 ml/min) (11–15, 39).

Nitrites Assay

The concentration of nitrites in the suffusate was deter-

mined by a modified Griess reaction as previously described

(31). Briefly, samples (1 ml) of the suffusate were incubated

with Escherichia coli nitrate reductase (0.5 U/ml) at room

temperature for 10 min to convert nitrates to nitrites. There-

after, concentration of nitrites was measured in duplicate by

mixing each sample with an equal volume (400 µl) of the

Griess reagent. The mixture was incubated at room tempera-

ture for 10 min, and absorbency was then measured at 540

nm using a spectrophotometer (SpectraMax 340; Molecular

Devices, Palo Alto, CA). The concentration of nitrites in each

sample (expressed as µM) was determined from a standard

curve obtained using known concentrations of NaN3 and

NaNO2 in distilled water. Nitrites concentration in buffer

was subtracted from experimental values.

cGMP Assay

The concentration of guanosine 3′,5′-cyclic monophosphate

(cGMP)-like immunoreactivity in the suffusate was deter-

mined 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-methylxan-

thine (1.0 mM) was added to samples to avoid possible interfer-

cence by changes in phosphodiesterase activity during

application of test compounds and to facilitate cGMP accumu-

lation. Optical density was read at 450 nm within 30 min at

room temperature using a thermoregulated ELISA micro-

plate 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 for-

mation 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 intrave-

nously, and the number of leaky sites and clearance of

FITC-dextran were determined for 30 min. Then, two concen-
	rations 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 inter-

vals 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 deter-

mined 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, suffu-

sion of saline for the entire duration of the experiment was

not associated with visible leaky site formation and increase

during clearance of FITC-dextran. The concentrations of bradyki-

nin and methotrexate used in these studies were based on

previous studies in our laboratory and reports in the litera-

ture (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 intrave-
nously, and the number of leaky sites and clearance of

FITC-dextran were determined for 30 min. Then, two concen-

trations 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

Nε-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 associ-

ated with visible leaky site formation and increase in clear-
ance 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, 31, 34).

In another series of experiments, the suffusate was col-
clected into prechilled polypropylene test tubes during the last
5 min of 30-min suffusion period of saline (control), methotrex-
ate (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 concentra-
tion and cGMP-like immunoactivity.

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 metalloen-
zymes, such as angiotensin I-convertase 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 cyclooxy-
genase 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, brady-
kinin (0.5 µM) was suffused for 5 min before and after meth-
отrexate 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 re-
sponses by determining its effects on adenosine-induced
leaky site formation and increase in clearance of FITC-
dextran from the cheek pouch. Adenosine increases macromo-
lecular efflux from the cheek pouch through a receptor-
mediated, NO-independent mechanism(s) (12, 15, 22, 39).
After bicarbonate buffer was suffused for 30 min (equili-
bration period), FITC-dextran 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 determin-
ing 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 ionomy-
cin (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 deter-
mined 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, adeno-
sine, ionomycin, Escherichia coli nitrate reductase, NaNO2,
NaNO3, 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 experi-
mental condition. Statistical analysis was performed using
two-way analysis of variance and the Newman-Keuls 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 $10 \pm 1.0/0.11 \text{ cm}^2$ during suffusion of bradykinin (0.5 µM) alone to $19 \pm 2.0/0.11 \text{ cm}^2$ 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) alone (Fig. 2A; each group, $n = 4$; $P < 0.05$). The number of leaky sites decreased significantly from $19 \pm 2.0/0.11 \text{ cm}^2$ during suffusion of 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 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 $6 \pm 1.0/0.11 \text{ cm}^2$ during suffusion of L-NAME (100 µM), methotrexate (5 mg/kg), and bradykinin (0.5 µM) to $23 \pm 2.0/0.11 \text{ cm}^2$ 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...
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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</thead>
<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>
</tr>
</tbody>
</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.

Nitrites 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 × 10⁻⁶ during suffusion...
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 bradykinin (0.5 µM) and was 22 ± 3/0.11 cm² after intravenous 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 significant effects on adenosine (0.5 µM)-induced leaky site formation and increase in clearance of FITC-dextran (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 adenosine (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 potentiates 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 indomethacin, 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.

Rubinstein and Mayhan (27) showed that suffusion of L-NAME onto the cheek pouch is associated with significant vasoconstriction. Hence, changes in vasomotor 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 vasomotor constriction. 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 nitrites 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 nitrites 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, nitrites 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 domains 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 ZnCl$_2$ to allow reincorporation of Zn$^{2+}$ 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.

This study was supported, in part, by grants from the National Institutes of Health (DE-00386) and a University of Illinois Scholar Award. Address for reprint requests: I. Rubinstein, Dept. of Medicine (M/C 787), Univ. of Illinois at Chicago, 840 South Wood St., Chicago, IL 60612-7323.

Received 13 November 1996; accepted in final form 23 May 1997.

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