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Complement Activation of Electrogenic Ion Transport in Isolated Rat Colon

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ABSTRACT. The complement cascade is an important component in many immune and inflammatory reactions and may contribute to both the diarrhoea and inflammation associated with inflammatory bowel disease. Isolated rat colonic mucosae were voltage clamped in Ussing chambers. Basolateral addition of zymosan-activated whole human serum (ZAS) induced a rapid onset, transient inward short circuit current (SCC). This response was concentration dependent and was significantly attenuated by pre-heating ZAS at 60°C for 30 min. Depletion of complement from normal human serum with cobra venom factor (CVF) significantly lowered SCC responses. Chloride was the primary charge carrying ion as responses to ZAS were abolished in the presence of the loop diuretic bumetanide. The complement component C3a stimulated ion transport but not to the same extent as whole serum. Exogenous C5 was without effect. The cyclooxygenase inhibitor piroxicam significantly attenuated the response to ZAS. These findings support the possibility that complement activation may contribute to the pathophysiology of secretory diarrhoea since activation of electrogenic chloride secretion converts intestinal epithelia to a state of net fluid secretion. BIOCHEM PHARMACOL **54**;10:1133–1137, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. complement; serum; ion transport; colon; intestine; diarrhoea

The complement system is composed of more than 20 serum proteins that interact in a precise series of enzymatic cleavage and membrane binding events leading to the generation of products with immunoprotective, immunoregulatory, and proinflammatory properties [1]. Complement can be activated through either of two distinct enzymatic cascades, referred to as the classical and alternative pathways. The classical pathway is generally initiated by the interaction of C1q with antibody/antigen complexes, whereas the alternative pathway is initiated by deposition of C3b on a variety of substrates including bacterial lipopolysaccharide and cell membranes. The formation of C3b is necessary for the amplification and progression of the complement cascade through both pathways.

Both the classical and alternative pathways converge at C5,§ which is cleaved to form a range of fluid-phase and membrane-phase products with multiple proinflammatory effects. C5a is the most potent anaphylatoxin, inducing alterations in smooth muscle and vascular tone, as well as vascular permeability [1]. It is also a powerful 'chemotaxin' and activator of both neutrophils and monocytes. C5a-mediated cellular activation can significantly amplify inflammatory responses by inducing the release of multiple additional inflammatory mediators, including hydrolytic enzymes, cytokines, arachidonic acid metabolites and reactive oxygen species. C5 cleavage also leads to the formation of C5b-9, or the membrane attack complex (MAC). There is now strong evidence that the MAC may play an important role in inflammation in addition to its role as a lytic pore-forming complex, as it also stimulates the release of many of the same proinflammatory molecules as C5a and promotes thrombosis following deposition on platelets and endothelium [2].

The aim of this investigation was to examine a potential role for complement or complement components in regulation of epithelial ion transport in *ex vivo* inflammatory conditions. We employed isolated sheets of epithelia with attendant lamina propria from rats. These tissues were exposed *in vitro* to human sera as a source of activated complement. Purified anaphylatoxins were also added to epithelia/lamina propria preparations. C9-deficient and reconstituted sera were used as stimuli to examine the possible contribution of MAC to the ion transport responses we found following challenge with whole (activated) sera.

MATERIALS AND METHODS

Non-fasted male Wistar rats (250–300 g) were killed by cervical dislocation. Segments of distal colon were opened

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§ *Abbreviations:* C3, third component of complement; C3a, fragment of C3; C5, C6, C9, fifth, sixth and ninth components of complement; CD, Crohn's disease; CVF, cobra venom factor; PGE_2, prostaglandin E_2; IBD, inflammatory bowel disease; LDH, lactate dehydrogenase; MAC, membrane attack complex; SCC, short circuit current; TTX, tetrodotoxin; UC, ulcerative colitis; ZAS, zymosan-activated whole human serum.

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and stripped of underlying smooth muscle by blunt dissection. Mucosal sheets of epithelium and attendant lamina propria were mounted in Ussing chambers (window area = 0.63 cm^2). Tissues, bathed on either side with Krebs Henseleit solution maintained at 37° and oxygenated with 95% O_2/5% CO_2, were voltage clamped to zero potential difference using a DVC 1000 (World Precision Instruments). The composition of the Krebs Henseleit solution was (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11.1 D-glucose. Short circuit current (SCC) was continuously monitored using an analogue to digital data acquisition system (MacLab, ADInstruments). Tissues were allowed to equilibrate until a steady state basal SCC was achieved. Drugs were added to either the apical (luminal) side or the basolateral (serosal) side bathing solutions. In some experiments the chloride containing salts of the Krebs–Henseleit solution were replaced (in mM) with sodium gluconate (117), potassium gluconate (4.7) and calcium sulphate dihydrate (2.5).

Human serum was prepared as described previously [3]. Serum complement was activated by incubating with 3.3 mg zymosan/mL serum for 20 min at room temperature. Zymosan was subsequently removed by centrifugation. To accommodate biological variability in different batches of sera which were prepared on different days, we designed paired experiments using test and control tissues obtained from a single animal, challenged with identical samples of freshly prepared sera.

LDH was assayed by a discontinuous method described elsewhere [4] using a Molecular devices V_{max} plate-reader. For LDH assays, segments of stripped epithelium (with attendant lamina propria) were maintained at 37° in oxygenated Krebs–Henseleit solution. These tissues were exposed to sera over a 10 min period. Assays were performed in duplicate. Tissue protein content was determined by the method of Lowry [5].

Drugs and Chemicals

Bumetanide, complement C3a, C5, C6 deficient serum, C9, C9 deficient serum, cobra venom factor, forskolin, LDH assay chemicals, PGE_2, piroxicam, and zymosan were all obtained from the Sigma Chemical Co.; PGE_2 RIA kit was obtained from Amersham International plc; all other chemicals were of analytical reagent grade.

Statistical Analysis

Paired preparations of mucosal segments from each rat were used throughout. Changes in ion transport (ΔSCC) are given as peak values. Results are expressed as mean ± SEM and statistical comparison was carried out using a two-tailed paired Student's *t*-test, Mann–Whitney test or by repeated measures analysis of variance where appropriate.

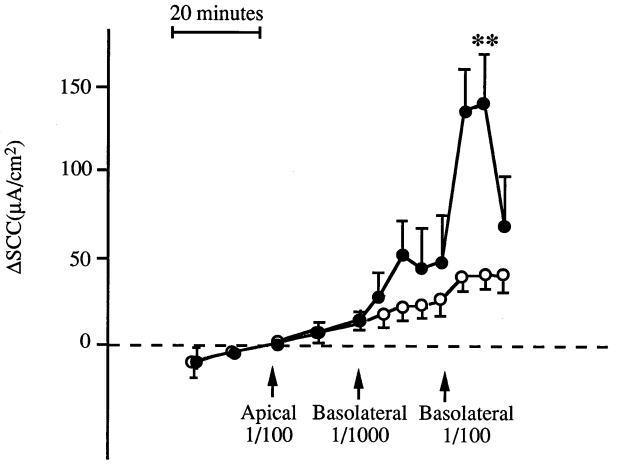

FIG. 1. Zymosan-activated human serum (ZAS, closed circles) caused a rapid onset, transient inward short circuit current. This response was concentration dependent and was not observed when ZAS was added to the apical bathing solution ($P < 0.005$; N = 7). Heat inactivation of ZAS (open circles) resulted in significantly lower SCC responses when compared with responses to ZAS in control tissues ($P < 0.005$; N = 7). Time is indicated by the horizontal bar.

RESULTS

Activated Serum Stimulates Electrogenic Ion Transport in Rat Colon

Basal short circuit current (66.2 ± 13.3 μA/cm^2; N = 7) and transepithelial resistance (124.3 ± 12.2 Ω.cm^2; N = 7) were stable over several hr following a 30 min equilibration period. Zymosan-activated human serum (ZAS) added to the basolateral bathing solution of voltage clamped rat colon produced an immediate onset, transient inward short circuit current (Fig. 1). The SCC response to serum was concentration dependent and was not observed when ZAS was added to the apical bathing solution. Normal, untreated human serum (1/100 dilution) also stimulated inward SCC (Fig. 2).

Nature of the Charge Carrying Ion(s)

The term inward SCC, by convention, is current which can be accounted for by secretion of anions, absorption of cations or by a combination of each. We attempted to identify the charge carrying ion pharmacologically by carrying out paired experiments with control responses to ZAS which were compared with responses to challenge in the presence of the loop-diuretic bumetanide (1 mM) added to the basolateral bathing solution. Bumetanide alone caused a fall in SCC of 40.3 ± 12.6 μA/cm^2 (N = 6). Ion transport responses to basolateral challenge with ZAS were virtually abolished in the presence of bumetanide (16.0 ± 4.9 μA/cm^2; $P < 0.05$; N = 6). Furthermore, SCC responses to the directly acting secretagogue forskolin were also attenuated by bumetanide from 126.4 ± 26.8 μA/cm^2 in controls to 36.4 ± 9.0 μA/cm^2 in bumetanide treated tissues.

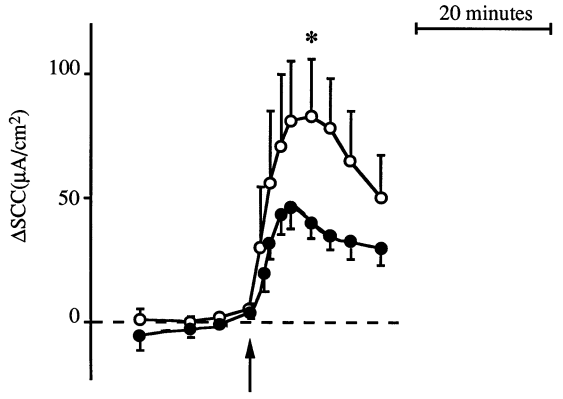

FIG. 2. Pre-treatment of normal human serum with cobra venom factor (CVF; 56 μM; closed circles) which exhausts normal reserves of complement proteins, reduced the capacity of serum to increase SCC when compared with responses to untreated serum ($P < 0.05$; N = 4; open circles). Sera, which was applied basolaterally, was used at 1/100 dilution throughout. Time is indicated by the horizontal bar.

There was a significant attenuation of ion transport responses to ZAS (1/100 dilution added basolaterally) when experiments were carried out in chloride-free bathing solutions (20.1 ± 5.6 μA/cm^2) compared with responses obtained in paired preparations bathed in normal Krebs–Henseleit solutions (156.6 ± 52.9 μA/cm^2; $P < 0.05$; N = 4). Similarly, SCC responses to the established chloride secretagogue forskolin (3 μM) were reduced from 181.9 ± 30.6 μA/cm^2 to 57.1 ± 18.2 μA/cm^2 in chloride free solutions ($P < 0.05$; N = 4).

Evidence that ZAS Evoked Changes in Ion Transport are Due to Complement Components

Heat inactivation of ZAS (60°C for 30 min) significantly attenuated SCC responses when compared with the effects of ZAS in paired, control tissues (Fig. 1). Depletion of complement components by treatment of normal human serum with cobra venom factor (CVF, 56 μM) significantly reduced ion transport responses (Fig. 2).

Complement Components Which May Contribute to Ion Transport Responses to Activated Sera

The complement component C3a induced inward current responses which were relatively modest when compared with SCC changes due to ZAS. C5a (activated C6-deficient serum containing exogenous C5) was without significant effect on SCC (Fig. 3). In order to examine whether biological activity of the cascade resides in downstream (post C6) components we used zymosan-activated C9-deficient sera which caused a stimulation of SCC which was enhanced by reconstitution of C9-deficient serum with exogenous C9 (7 μM; Fig. 4).

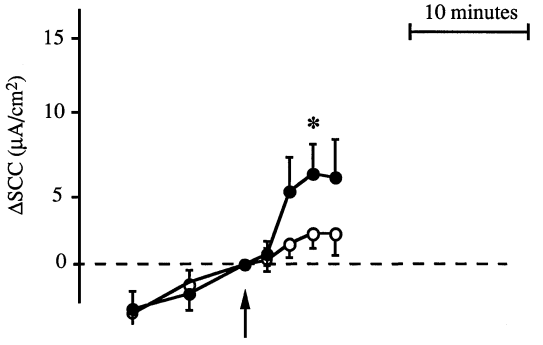

FIG. 3. Complement component C3a (1 μM, closed circles) induced a significant increase in short circuit current in response to basolateral challenge when compared with activated C5 (4 μM, open circles) which failed to stimulate ion transport when background electrical drift of the tissue was taken into account ($P < 0.05$; N = 8). Time is indicated by the horizontal bar.

Cellular Targets for Complement Activation of Epithelial Ion Transport

Ion transport responses to ZAS (130.2 ± 14.1 μA/cm^2) were significantly reduced by pre-treatment of tissues with the cyclooxygenase inhibitor piroxicam (15.9 ± 3.8 μA/cm^2; $P < 0.05$; N = 6). In contrast, responses to exogenous PGE_2 were increased from 61.9 ± 13.2 μA/cm^2 to 113 ± 16.5 μA/cm^2 in the presence of piroxicam ($P < 0.05$; N = 6). In separate experiments, we showed that exposure of mucosal sheets to ZAS evoked measurable release of PGE_2 and that this effect was also sensitive to piroxicam (Fig. 5). Piroxicam had no significant effect on basal PGE_2 release, with levels measuring 50 ± 6.3 pg/mg protein (N = 12) in controls as compared with 32.1 ± 11.3 pg/mg protein (N = 11) in the presence of piroxicam.

Effects of ZAS Upon Parameters Which Reflect Toxicity

ZAS treatment did not impair the capacity of tissues to respond to the directly acting secretagogue forskolin. Responses to forskolin (30 μM) were 70.4 ± 22.4 μA/cm^2 (N = 6) in control tissues compared with 105.7 ± 30

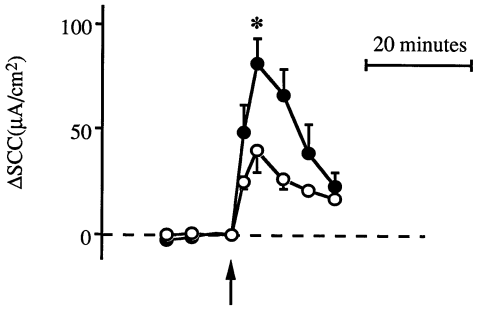

FIG. 4. Zymosan-activated C9-deficient sera, added basolaterally (open circles), caused a stimulation of SCC which was significantly enhanced by reconstitution of C9-deficient serum with exogenous C9 (7 μM; $P < 0.05$; N = 7; closed circles). Time is indicated by the horizontal bar.

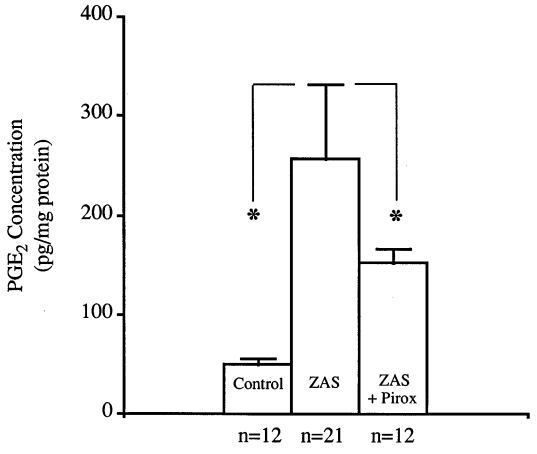

FIG. 5. Incubation of colonic mucosa with activated serum (1/100 dilution), resulted in the release of significantly higher concentrations of PGE_2 compared with control tissues ($P < 0.05$), and tissues treated with activated serum in the presence of piroxicam (10 μM; $P < 0.05$).

$\mu A/cm^2$ in ZAS treated tissues (N = 7). Increased LDH release was a feature of treatment of isolated tissues with C3a and C5 (Fig. 6) over a similar time course to that used in ion transport studies. ZAS treatment did not significantly elevate LDH release above basal levels.

DISCUSSION

Zymosan-activated sera caused an immediate onset ion transport response in voltage clamped colonic epithelia. ZAS was without effect when added to the apical bathing solution, indicating an effect upon the basolateral domain of epithelial cells or alternatively upon non-epithelial targets within the lamina propria. The SCC response is accounted for, at least in part, by electrogenic chloride secretion since it was reduced by the loop diuretic bumetanide which blocks the basolateral Na/K/Cl co-transporter upon which electrogenic chloride secretion depends [6]. Further evidence for electrogenic chloride secretion was

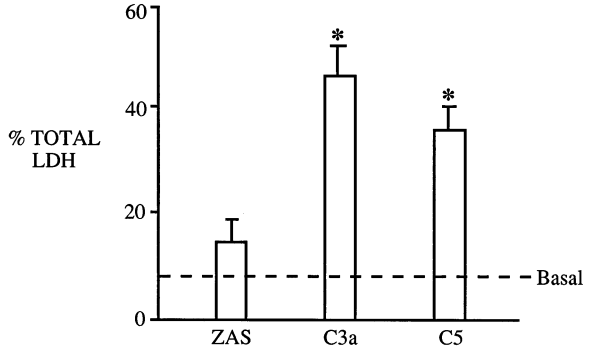

FIG. 6. Levels of lactate dehydrogenase (LDH) release expressed as % of total cell LDH were elevated following treatment of isolated tissues with C3a (1 μM) and C5a (4 μM). ZAS treatment (1/100 dilution), did not significantly elevate LDH release above basal levels. Basal levels of LDH release are indicated by a dashed line. (*$P < 0.05$; N = 6).

obtained from ion substitution studies. Replacement of chloride reduced responses to ZAS and to the established secretagogue forskolin. Chloride secretion establishes an electrochemical gradient which, *in vivo*, alters the colon from an absorptive tissue to one of net fluid secretion. Thus the events we have measured may underlie a process which leads to secretory diarrhoea.

Several pieces of evidence implicate complement involvement in stimulation of ion transport. First, we used heat treatment (56°–60° for 30 min) to inactivate both classical and alternative pathways of complement activation by denaturation of heat labile proteins [7]. The results showed the action of ZAS to be heat-sensitive, consistent with an effect mediated by complement. Secondly, stimulation of SCC by normal sera was reduced by pre-treatment of sera with cobra venom factor which depeletes serum lytic potential by overstimulating the complement cascade [8]. It is quite possible that the residual response to ZAS following either heat inactivation or cobra venom factor treatment is due to complement–independent factors in the sera we used.

Exogenous C3a, but not C5, caused inward SCC responses although the magnitude of change was less than that observed in response to ZAS. It is tempting to speculate as to why ZAS is more effective than C3a and C5 with regard to effects on SCC whereas C3a and C5 are more effective than ZAS at elevating LDH levels. Zymosan addition to serum will produce significant quantities of active C3bBb, which permit MAC formation to persist after zymosan removal. MAC insertion into target cell membranes is accompanied by sustained Ca^{2+} influx (3) which will activate Ca^{2+} sensitive Cl^- channels. The anaphylatoxins, however, may exert an indirect effect via stimulation of superoxide production from local phagocytic cells [1]. The superoxide may be responsible for cell damage and death in the preparation which is detected as LDH release.

The concentrations of the anaphylatoxins we used were 1 μM C3a and 4 μM C5a which correspond to 'normal' values [9]. Complement activation, by either the classical pathway or the alternative pathway, leads to the generation of products that not only maintain normal host defence integrity but also result in inflammation and tissue destruction. Although much more work needs to be done, our results indicate that anaphylatoxins, which are less effective than ZAS at stimulating ion transport, are more effective than ZAS at causing LDH release.

Taken overall, our observations indicate that much of the capacity of the cascade to stimulate electrogenic secretion resides in downstream (post C6) components. This hypothesis is further supported by the observation that activated C9-deficient serum was pro-secretory in this preparation. Reconstitution with exogenous C9 enhanced the pro-secretory activity of C9-deficient serum. These observations are consistent with membrane-phase components (MAC) of the complement cascade being principally involved in stimulation of ion transport, with perhaps a

lesser contribution from the fluid phase anaphylatoxins. Although increased release of LDH was a feature, it is interesting to note that ion transport responses to the directly acting secretagogue forskolin were not reduced following exposure of tissues to ZAS.

Arachidonic acid metabolites may act as mediators of the actions of complement on this preparation. Ion transport responses were reduced by pretreatment of tissues with the cyclooxygenase inhibitor piroxicam. PGE_2 formation was a feature of exposing mucosal tissues to ZAS and this was also sensitive to piroxicam, although piroxicam failed to abolish either the effect of ZAS upon SCC or the effect of ZAS upon PGE_2 generation. That prostaglandins may mediate or modulate other tissue responses to complement has already been described [10, 11]. The intracellular increase in Ca^{2+} concentration occuring due to late phase complement component insertion into cell plasma membrane could stimulate PLA_2 activity, giving rise to elevated arachidonic acid concentrations and hence prostaglandin production rates.

Remarkably, complement contribution to intestinal disease, including inflammatory bowel disease (IBD), has not been extensively studied to date. In this area, there have been several provocative studies which report differences between Crohn's disease (CD) and ulcerative colitis (UC) [12]. Complement levels are also known to vary with disease activity, supporting the possibility that the complement system could play a role in IBD etiology or pathophysiology [13–16]. Our experiments, carried out *in vitro* and in the absence of blood-borne factors, indicate that while complement effects upon vasculature may contribute to disease [17, 18] direct effects upon other tissue types may also be important.

The complement cascade therefore represents an important but complex component of the immune system and may play a critical role in the manifestation of certain inflammatory conditions in the gut. The development of therapeutic agents targeted against specific components of the complement cascade may prove of value in prevention, or cure, of the inflammation and diarrhoea associated with inflammatory diseases of the gastrointestinal tract.

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