The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 2005

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J Immunol (2005) 174 (4): 2235–2241.
https://doi.org/10.4049/jimmunol.174.4.2235

A Proinflammatory Peptide from Herpes Simplex Virus Type 2 Glycoprotein G Affects Neutrophil, Monocyte, and NK Cell Functions[1]

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We have identified a synthetic peptide derived from the secreted portion of HSV type 2 glycoprotein G, denoted gG-2p20, which has proinflammatory properties in vitro. The gG-2p20 peptide, corresponding to aa 190–205 of glycoprotein G-2, was a chemoattractant for both monocytes and neutrophils in a dose-dependent fashion, and also induced the release of reactive oxygen from these cells. The receptor mediating the responses was identified as the formyl peptide receptor. The gG-2p20-induced activation of phagocytes had a profound impact on NK cell functions. The reactive oxygen species produced by gG-2p20-activated phagocytes both inhibited NK cell cytotoxicity and accelerated the apoptotic cell death in NK cell-enriched lymphocyte populations. Hence, we have for the first time been able to identify a potential function of the secreted portion of HSV-2 glycoprotein G. We propose that the proinflammatory gG-2p20 peptide identified could contribute to a reduced function and viability of NK cells during HSV-2 infection due to its ability to recruit and activate phagocytic cells. *The Journal of Immunology,* **2005, 174: 2235–2241.**

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects the genital tract mucosa and is the most common causative agent of genital ulcer disease in humans. Once HSV-2 infects the epithelium and replicates, it can be transmitted to the sacral ganglia via nerve axons and establish a latent infection, which in association with factors such as, for example, stress, fever, and immunosuppression, can reactivate and cause recurrent disease.

The HSV-2 genome codes for 11 different glycoproteins. Glycoprotein G is the least conserved vis-à-vis the closely related HSV-1, with a sequence homology of <30%. The glycoprotein G from HSV-2 (gG-2) is 699 aa in length and is the only of the HSV envelope proteins that is cleaved posttranslational during processing, resulting in a secreted N-terminal protein (sgG-2) of ~300 aa in length, and a C-terminal protein constituting the cell membrane-anchored gG-2 (1–4). The sgG-2 protein is unique for HSV-2, but it has not yet been ascribed a specific function.

NK cells and phagocytes (neutrophils and monocytes) constitute an early line of defense against most viral infections. NK cells have been shown to be particularly important in HSV infections; a resounding theme of NK cell deficiencies is the susceptibility to herpes virus infections, including HSV-2 (5–9). The particular susceptibility of HSV-infected cells to NK cell-mediated killing is a result of the virus-induced down-regulation of MHC class I expression on HSV-infected cells (10, 11). NK cells are also a rich source of IFN-γ, which contributes to their antiviral effect (12).

Also, neutrophils and monocytes are involved in limiting and clearing HSV-2 vaginal infections. Mice depleted of polymorphonuclear leukocytes are less able to control genital viral replication (13, 14). A patient with reduced polymorphonuclear leukocytes and monocyte function who suffered from severe and recurrent HSV-2 genital infection was successfully treated by GM-CSF injections, as this treatment was associated with a restored function of the impaired cells (15). Similarly, treatment of guinea pigs with M-CSF, which specifically enhances the monocyte population, protected these animals against a primary HSV-2 infection (16).

Neutrophils and monocytes are directed to infected tissues by chemotaxis. Numerous chemoattractants have been identified, both of bacterial and endogenous origin. These include the classical chemoattractants such as *N*-formyl-methionyl-leucyl-phenylalanine (fMLF),[3] activated complement component 5, leukotriene B_4, platelet-activating factor, and IL-8 (17). All of these agonists work through seven-transmembrane-spanning, G protein-coupled receptors (GPCR), of which the formyl peptide receptor (FPR) is one of the most thoroughly studied. The FPR belongs to the FPR family, which also includes the homologues FPR-like-1 (FPRL1) and -2 (FPRL2). FPR and FPRL1 are expressed on both neutrophils and monocytes, but monocytes alone also express FPRL2 (18). The most investigated FPR ligands, the *N*-formylated peptides, are cleavage products of bacterial and mitochondrial proteins, and serve as powerful attractants for mammalian phagocytes to infected and damaged tissue (19). Recently, however, a number of novel agonists and antagonists have been identified for FPR and its variants FPRL1 and FPRL2, including peptide domains derived from HIV-1 envelope proteins, small synthetic peptides derived from random peptide libraries, and host-derived peptide or lipid agonists (17, 20, 21).

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Received for publication October 26, 2004. Accepted for publication December 6, 2004.

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[1] These studies were supported by the Swedish Science Council (Medicine).

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[3] Abbreviations used in this paper: fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; CL, luminol-ECL; FPR, formyl peptide receptor; GPCR, G protein-coupled receptor; *N-t*-Boc-MLF, *N-t*-butoxycarbonyl-methionine-leucine-phenylalanine; ROS, reactive oxygen species; TP3, To-Pro-3.

0022-1767/05/$02.00

The FPR family ligands are also potent activators of the phagocyte NADPH-oxidase, an enzyme system responsible for the production of reactive oxygen species (ROS). Upon activation of the NADPH-oxidase, electrons are transferred from cytosolic NADPH to molecular oxygen on the opposite side of the cell membrane, resulting in production and release of superoxide anion. This very reactive molecule is rapidly converted to hydrogen peroxide and other toxic oxygen products. Although the ROS released from activated leukocytes are toxic to many pathogens, they are also damaging to the host itself, leading to an enhanced inflammatory reaction and tissue destruction. Released ROS can also affect the function of adjacent cells, e.g., NK cells, leading to reduced cytotoxic killing potential of these cells (22, 23).

We have investigated the in vitro inflammatory properties of sgG-2 using synthetic peptides spanning the entire amino acid sequence of sgG-2. An initial screening revealed that one peptide, gG-2p20 (aa 190–205), induced an intense oxidative burst in neutrophils. Desensitization experiments and specific receptor blockage revealed that gG-2p20 acted through the FPR. The gG-2p20 peptide was shown to serve as a chemoattractant for both neutrophils and monocytes and also as an activator of the NADPH-oxidase in both of these cell types. We could also show that phagocytes activated by gG-2p20 suppressed the function of NK cells by inhibiting their cytotoxic potential and accelerating their apoptosis. This inhibitory event was mediated by oxygen radicals. Our results suggest that gG-2p20 could contribute to the accumulation and activation of neutrophils and monocytes during HSV-2 infection. Furthermore, gG-2p20 could be involved in down-regulating the numbers of functional NK cells during acute HSV-2 infection.

Materials and Methods

Peptides and reagents

Thirty-three 15-mer synthetic peptides, with 5 overlapping aa to each end (Table I) covering the entire secreted portion of the HSV-2 glycoprotein G (sgG-2) of strain HG52 (24), were synthesized by M. Levi (Kardinska Institute, Stockholm, Sweden) , as described earlier using F-moc synthesis chemistry (25). The purity of the peptides was found to be ≥85% in reverse-phase HPLC. The HSV-2 gG-2 peptides were dissolved in either H_2O or sodium bicarbonate (0.02 M $NaHCO_3$) to 1 or 5 mg/ml (gG-2p20), and stored at −70°C until use.

The hexapeptide WKYMVM was synthesized and HPLC purified by K. Ross-Petersen (Holte, Denmark), dissolved in DMSO (0.01 M DMSO) to 10 mM, and stored at −70°C until use. The formylated tripeptide fMLF was purchased from Sigma-Aldrich, dissolved in DMSO to10 mM, and stored at −70°C until use. Subsequent dilutions of all peptides were made in Krebs-Ringer phosphate buffer (pH 7.3; 120 mM NaCl, 5 mM KCl, 1.7 mM KH_2PO_4, 8.3 mM NaH_2PO_4, and 10 mM glucose) supplemented with Ca^{2+} (1 mM) and Mg^{2+} (1.5 mM).

Ficoll-Hypaque was obtained from Amersham Biosciences. *N-t*-butoxycarbonyl-methionine-leucine-phenylalanine (*N-t*-Boc-MLF), luminol, and PMA were obtained from Sigma (Sigma-Aldrich), and HRP and catalase were obtained from Boehringer Mannheim.

Isolation of human neutrophils

Human peripheral blood neutrophils were isolated from buffy coats from healthy blood donors (Blood Centre, Sahlgrenska University Hospital) using dextran sedimentation and Ficoll-Paque gradient centrifugation, as described (26). The cells were washed and resuspended in Krebs-Ringer phosphate buffer and stored on melting ice until use.

Isolation of human lymphocytes and monocytes

Peripheral venous blood was obtained as freshly prepared leukopacks from healthy blood donors (Blood Centre, Sahlgrenska University Hospital). After Ficoll-Hypaque centrifugation, mononuclear cells were separated into lymphocytes and monocytes using countercurrent centrifugal elutriation, as described in detail elsewhere (22). This procedure yielded a fraction with >90% monocytes at a flow rate of ~19 ml/min, and a lymphocyte fraction enriched for NK cells ($CD3^-/CD56^+$ phenotype) (45–50%) and T cells ($CD3^+/CD56^-$ phenotype) (35–40%) at a flow rate of 14–15 ml/min. Pure preparations of NK cells were obtained using the NK isolation kit II from Miltenyi Biotec, according to the manufacturer's instructions.

Chemiluminescence measurements

The NADPH-oxidase activity was determined using luminol-ECL (CL) (27). The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Technologies), using disposable 4-ml polypropylene tubes with a 400-μl reaction mixture containing 4×10^4 cells. The tubes were equilibrated in the Biolumat for 5 min at 37°C, after which the stimulus (20 μl) was added. The light emission was recorded continuously and is depicted as cpm. By a direct comparison of the superoxide dismutase-inhibitable reduction of cytochrome *c* and superoxide dismutase-inhibitable CL, 7.2×10^7 counts were found to correspond to production of 1 nmol superoxide (a millimolar extinction coefficient for cytochrome *c* of 21.1 was used). When experiments were performed with the antagonist *N-t*-Boc-MLF, this substance was included in the reaction mixture during the equilibrium period.

Neutrophil and monocyte chemotaxis and NADPH-oxidase activity

Neutrophil and monocyte chemotaxis was determined using ChemoTX multiwell chambers (NeuroProbe) with membrane pore diameters of 3 and

Table I. *The amino acid sequence of 33 synthetic peptides covering the entire sgG-2 protein, and the oxidative responses induced by these peptides in neutrophils*

Peptide	Sequence[a]	O_2^- Prod.	Peptide	Sequence	O_2^- Prod.
1	$MHAIAPRLLLLFVLS_{15}$	−	18	$WDRAAETFEYQIELG_{185}$	−
2	$LFVLSGLPGTRGGSG_{25}$	−	19	$QIELGGELHVGLLWV_{195}$	−
3	$RGGSGVPGPINPPNS_{35}$	−	20	$GLLWVEVGGEGPGPT_{205}$	++
4	$NPPNSDVVFPGGSPV_{45}$	−	21	$GPGPTAPPQAARAEG_{215}$	−
5	$GGSPVAQYCYAYPRL_{55}$	−	22	$ARAEGGPCVPPVPAG_{225}$	−
6	$AYPRLDDPGPLGSAD_{65}$	−	23	$PVPAGRPWRSVPPVW_{235}$	−
7	$LGSADAGRQDLPRRV_{75}$	−	24	$VPPVWYSAPNPGFRG_{245}$	−
8	$LPRRVVRHEPLGRSF_{85}$	−	25	$PGFRGLRFRERCLPP_{255}$	−
9	$LGRSFLTGGLVLLAP_{95}$	−	26	$RCLPPQTPAAPSDLP_{265}$	−
10	$VLLAPPVRGFGAPNA_{105}$	−	27	$PSDLPRVAFAPQSLL_{275}$	−
11	$GAPNATYAARVTYYR_{115}$	−	28	$PQSLLVGITGRTFIR_{285}$	−
12	$VTYYRLTRACRQPIL_{125}$	−	29	$RTFIRMARPTEDVGV_{295}$	−
13	$RQPILLRQYGGCRGG_{135}$	−	30	$EDVGVLPPHWAPGAL_{305}$	+
14	$GCRGGEPPSPRTCGS_{145}$	−	31	$APGALDDGPYAPFPP_{315}$	−
15	$KTCGSYTYTYQGGGP_{155}$	−	32	$APFPPRPRFRRALRT_{325}$	−
16	$QGGGPPTRYALVNAS_{165}$	−	33	$RALRTDPEGVDPDVR_{335}$	−
17	$LVNASLLVPIWDRAA_{175}$	−			

[a] The peptide sequences are overlapping by 5 aa in each end.
[b] Oxidative responses are given as − (peak value $<10^7$ cpm), +(1×10^7–5×10^7 cpm), and ++ (>5×10^7 cpm).

5 μm for neutrophils and monocytes, respectively, according to instructions provided by the manufacturer. Cells were allowed to migrate through the membranes during a 90-min incubation at 37°C, whereafter the cells were lysed with 0.2% cetyltrimethylammonium bromide in PBS-0.2% BSA. A total of 20 μl of each lysate was transferred into a 96-well ELISA plate (MaxiSorp; Nunc) and quantified based on myeloperoxidase activity by the addition of 45 μl of peroxidase substrate solution containing 0.1 mg/ml tetramethylbenzidine and 0.04% H_20_2 in 0.2 M phosphate-citrate buffer, pH 5. The reaction was stopped after 20 min with 25 μl of 1 M H_2SO_4, and the absorbance was measured at 450 nm. Relative migration (%) was obtained by dividing the sample OD_{450} with the OD_{450} value of cells added directly to the lower compartment of the multiwell chamber (representing 100% migration). Spontaneous movement, i.e., OD_{450} values obtained from samples with no added stimulus, was used as negative control. As positive control, migration toward fMLF (10^{-8} M) was used. The OD_{450} values obtained are means from duplicate wells.

Assay of NK cell cytotoxicity

A novel cytotoxicity assay using PKH-26-labeled K562 cells was used for studying NK cell cytotoxic function (28). For labeling, K562 cells were incubated with PKH-26 (5 μM) for 4 min at room temperature. The labeling reaction was terminated by the addition of serum, and the cells were washed thoroughly and resuspended in culture medium. The labeled K562 cells were incubated for 4 h with NK cells at an E:T ratio of 5:1 in 96-well microtiter plates (Nunc) in the presence or absence of neutrophils. Where indicated, catalase was added at the onset of incubation, and gG-2p20 was added after 20 min. After the 4-h incubation, the viability stain To-Pro-3 (TP3; Molecular Probes) was added (1 μM) and the cells were analyzed by flow cytometry. Lysed target cells were identified as double-positive, PKH-26^+, $TP3^+$ cells, while live K562 cells were TP3 negative. Results are presented as percentage of specific lysis: percentage of specific lysis = $100 \times$ (experimental percentage of $TP3^+$ K562 − spontaneous percentage of $TP3^+$ K562)/(100 − spontaneous percentage of $TP3^+$ K562).

Apoptosis in lymphocytes

NK cell-enriched lymphocytes were incubated with autologous gG-2p20-stimulated monocytes or neutrophils. After 16 h, lymphocytes were assayed for apoptosis by use of flow cytometry. End-stage apoptotic cells were identified as lymphocytes with altered scattering properties, i.e., reduced forward scatter and increased right angle scatter, as previously described (29). Lymphocyte apoptosis, after exposure to ROS-producing phagocytes, was confirmed using FITC-labeled annexin V (BD Pharmingen), according to the manufacturer's instructions.

Statistical method

Statistical analyses were done by ANOVA with Bonferroni's post test.

Results

An HSV-2 glycoprotein G-derived synthetic peptide induces phagocyte NADPH-oxidase activity

First, we investigated the oxygen radical production in neutrophils in response to 33 synthetic peptides spanning the whole amino acid sequence of sgG-2. In the primary screening, only two peptides, peptides 20 and 30, induced an oxidative burst in neutrophils. One of these peptides, peptide 20, corresponding to amino acid sequence 190–205 (Table I; peptide 20), induced superoxide anion production of a magnitude similar to that induced by the known phagocyte agonist fMLF (10^{-7} M), while peptide 30 gave much less activity. We denoted the activating peptide gG-2p20. Experiments were performed with gG-2p20 peptide ordered from two different companies, to confirm the results.

The NADPH-oxidase activity in response to gG-2p20 is pertussis toxin sensitive

The gG-2p20 peptide induced a robust oxygen radical production in neutrophils (Fig. 1). The ROS activation induced by this peptide was also shown to be dose dependent (data not shown). The time course and magnitude of the response resembled that induced by the well-defined neutrophils agonist fMLF (Fig. 1, *inset*). The oxidative response induced by gG-2p20 was abolished by preincubation of neutrophils with pertussis toxin, known to specifically

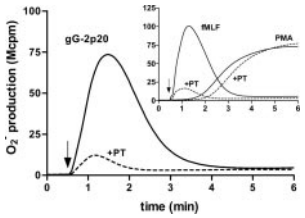

FIGURE 1. gG-2p20-induced activation of NADPH-oxidase in neutrophils. Neutrophils were preincubated at 37°C for 90 min in the absence (solid line) or presence (dotted line) of pertussis toxin (PT, 500 ng/ml), and were then stimulated with gG-2p20 (50 μg/ml). The superoxide anion release was measured continuously for 6 min by CL (27). To monitor the efficiency of the PT treatment, a PT-sensitive (fMLF, 10^{-7} M) and a PT-insensitive (PMA, 5×10^{-8} M) stimulus were used (*inset*). The amount of superoxide anion is presented as cpm. Arrows indicate the addition of agonist. One representative experiment of three is shown.

block signaling induced by seven-transmembrane-spanning receptors linked to G_i-type heterotrimeric G proteins (GPCR) (19). This result indicates that gG-2p20-induced activation is dependent on receptor binding involving a pertussis toxin-sensitive GPCR.

gG-2p20 is chemotactic for both neutrophils and monocytes

Because all known GPCR in phagocytes (e.g., FPRs, C5a receptor, PAF receptor, etc.) are chemotactic receptors, we investigated whether the HSV peptide had the capacity to induce phagocyte migration. The gG-2p20 peptide was found to be chemotactic for both neutrophils and monocytes (Fig. 2, *A* and *B*, respectively) in a dose-dependent fashion. The maximal transmigration observed

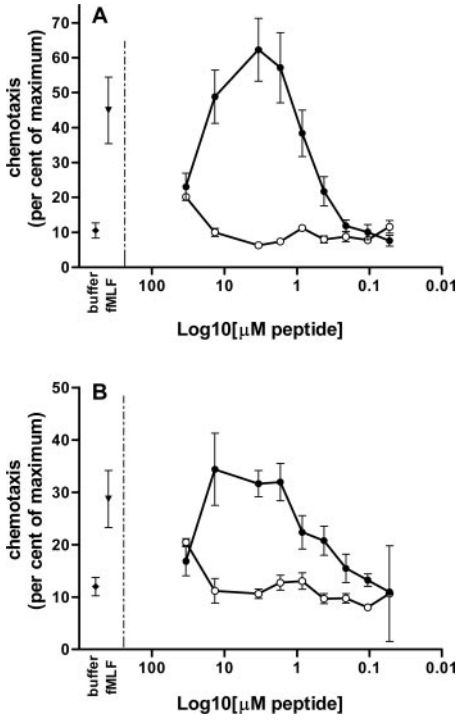

FIGURE 2. gG-2p20-induced chemotaxis in neutrophils and monocytes. Neutrophil (*A*) and monocyte (*B*) transmigration in response to gG-2p20 (●), control peptide gG-2p18 (○), buffer (rhomb), and fMLF (10^{-8} M, triangle) was determined using a ChemoTX multiwell chamber system. Migration was determined after 90-min incubation by measuring the amount of myeloperoxidase activity derived from the migrated cells. Data are expressed as the mean relative migration ± SEM achieved for different peptide concentrations ($n = 3$).

was comparable to that induced by the well-characterized neutrophil and monocyte chemoattractant fMLF (Fig. 2). The maximal neutrophil transmigration was achieved at a concentration of 3 μM gG-2p20, and a migratory effect could be seen at doses down to 0.2 μM. The maximal monocyte transmigration was shown at a concentration of 14 μM gG-2p20, and transmigration was evident down to a 0.05 μM concentration of the peptide. The EC_{50} values were 0.69 μM for neutrophils and 0.55 μM for monocytes.

gG-2p20 activates neutrophils and monocytes through FPR

The HSV peptide-induced response closely resembled responses induced by peptides (bacterial and synthetic) acting through the FPR family. We thus investigated the involvement of FPR, FPRL1, and FPRL2 in the responses induced by the HSV peptide. This was achieved by desensitization experiments using the gG-2p20, the FPR agonist fMLF, and the FPRL1/FPRL2 agonist WKYMVM (a synthetic hexapeptide). The basis for these experiments is the fact that stimulation of a GPCR due to agonist binding results in inactivation of the receptor and subsequent inability to respond to a second (or the same) agonist (18).

Attempting to restimulate phagocytes first activated with gG-2p20 with the same agonist showed that the peptide acts through a receptor that can be desensitized, i.e., no response was achieved after restimulation (homologous desensitization; Fig. 3*A* for neutrophils, and Fig. 3*D* for monocytes). Neither were cells that first were activated with the FPR agonist fMLF able to mount a second oxidative response when stimulated with gG-2p20 (Fig. 3*B* for neutrophils, and Fig. 3*E* for monocytes), i.e., fMLF desensitized the cells to the HSV peptide (heterologous desensitization). In contrast, no desensitization of the gG-2p20-induced activation occurred in cells first stimulated with the FPRL1/FPRL2 agonist WKYMVM (Fig. 3*C* for neutrophils, and Fig. 3*F* for monocytes). These results indicate that the HSV peptide uses FPR, but not FPRL1 (neutrophils and monocytes) or FPRL2 (monocytes), for activation of the phagocytes. In line with these results, we also found that gG-2p20 desensitized the response to FPR, but not to WKYMVM (data not shown). Furthermore, preincubation of the cells with the FPR antagonist *N-t*-Boc-MLF completely abrogated the gG-2p20-induced response (Fig. 4*A* for neutrophils, and Fig. 4*B* for monocytes). We thus conclude that FPR functions as a receptor for the HSV peptide, both in monocytes and neutrophils.

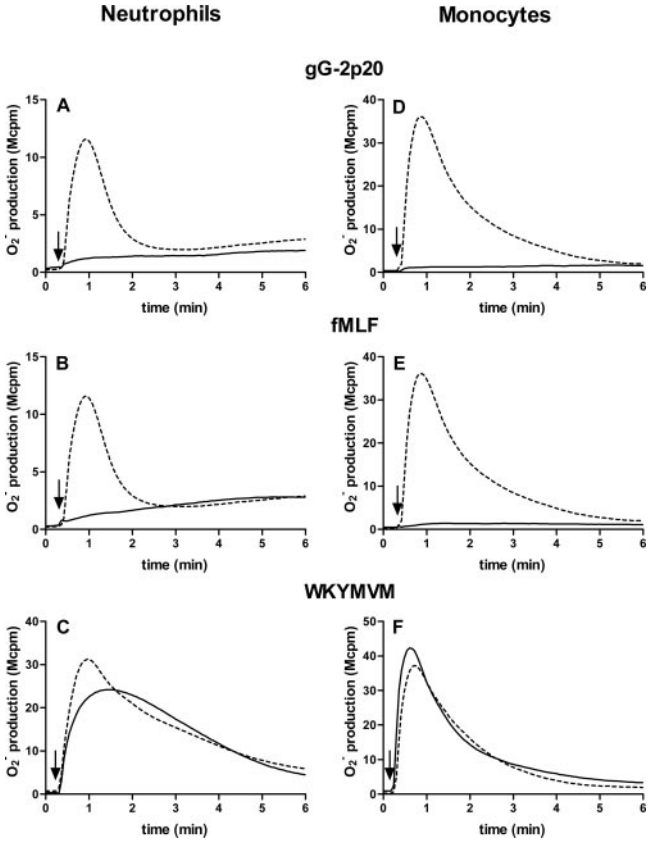

FIGURE 3. Desensitization of the gG-2p20 response. Neutrophils (*A–C*) and monocytes (*D* and *E*) were preactivated with one stimulus and then reactivated with another (or the same) to determine the desensitization pattern for the receptors/agonists. The primary stimulus was added to the reaction mixtures on ice, whereafter the cells were transferred to the luminometer and incubated at 37°C for 10 min before addition of the second stimulus. *A* and *D*, The primary stimulus was gG-2p20 (50 μg/ml), and the secondary stimulus gG-2p20 (50 μg/ml). *B* and *E*, The primary stimulus was fMLF (0.1 μM) (ligand for FPR), and the secondary stimulus gG-2p20 (50 μg/ml). *C* and *F*, The primary stimulus was WKYMVM (ligand for FPRL1 and FPRL2), and the secondary stimulus gG-2p20 (50 μg/ml). The amount of superoxide anion is presented as cpm. Arrows indicate addition of the second stimulus. The figures show one representative experiment of three.

Neutrophils activated by gG-2p20 down-modulate the NK cell cytotoxic function

Because ROS released from phagocytes are known to possess the capacity of impairing the function of NK cells, we measured the ability of NK cells to kill target cells in the presence of gG-2p20-stimulated neutrophils. Coincubation of NK cells with unstimulated neutrophils reduced the cytotoxic activity of the NK cells (Fig. 5). However, the suppressive effect on NK cytotoxicity was significantly increased when the coincubated neutrophils were stimulated with gG-2p20. This effect was reversed if the ROS-scavenger catalase was added to the cultures (data not shown), indicating that this effect was mediated by oxygen radicals. The gG-2p20 peptide by itself (i.e., in the absence of neutrophils) did not affect the cytotoxic potential of the NK cells (Fig. 5).

Neutrophils activated by gG-2p20 induce NK cell apoptosis

The ability of gG-2p20-activated neutrophils to impair the cytotoxic killing potential of NK cells led us to investigate whether the viability of lymphocytes was affected by ROS released from neutrophils. We found that gG-2p20 triggered substantial lymphocyte apoptosis, as manifested by an increased number of cells displaying morphological changes, indicating apoptotic decomposition after overnight incubation (Fig. 6). The gG-2p20 effect on lymphocyte apoptosis was clearly mediated by NADPH-oxidase-derived ROS, as lymphocytes were protected by the oxidase inhibitor diphenylene iodonium (data not shown) and the ROS scavenger catalase (Fig. 6).

Discussion

A proinflammatory peptide derived from the HSV-2 glycoprotein G, denoted gG-2p20, has been identified as a potent phagocyte chemoattractant and an inducer of ROS. This is to our knowledge the first suggested function for the secreted portion of HSV-2 glycoprotein G. The gG-2p20 peptide was shown to act through the phagocyte receptor FPR, and induced chemotactic responses and release of ROS to levels comparable to that induced by the known phagocyte chemoattractant fMLF. We could also show that the ROS secreted in response to gG-2p20 had a profound effect on the function of adjacent NK cells. The affected NK cells lost their ability to kill permissive target cells and also exhibited elevated apoptotic cell death.

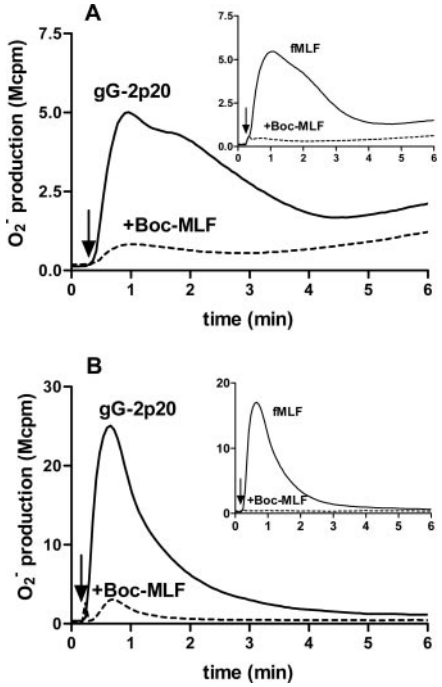

FIGURE 4. Inhibition of the gG-2p20-induced ROS response using *N-t*-Boc-MLF. Neutrophils (*A*) and monocytes (*B*) were preincubated at 37°C for 5 min in the absence (solid line) or presence (dotted line) of *N-t*-BOC-MLF (50 μM) and subsequently stimulated with gG-2p20 (50 μg/ml) or fMLF (0.1 μM; *inset*). The amount of superoxide anion is presented as cpm. Arrows indicate the addition of peptide. The figures show one representative experiment of three.

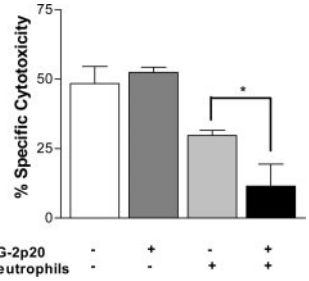

FIGURE 5. Neutrophil-dependent inhibition of NK cell cytotoxic function is induced by gG-2p20. PKH-26-labeled K562 cells were incubated for 4 h with NK cells at an E:T ratio of 5:1 in the presence or absence of neutrophils and gG-2p20. The viability stain To-Pro-3 was added, and the cells were analyzed by flow cytometry. Lysed target cells were identified as double-positive, PKH-26$^+$, TP3$^+$ cells. Results are presented as the mean percentage of specific cell lysis ± SEM (n = 5).

The FPR family has diverse and complex roles in biology, ranging from, for example, antimicrobial defense, regulation of inflammatory and anti-inflammatory processes, and involvement in amyloidogenic diseases (20). There is no discernible pattern for which FPR that is used in which process, but there are instead a great variability and overlap in agonist specificity. However, it is clear that the FPRL1 is the most promiscuous of the three receptors, to date known to bind 16 different agonists of bacterial, viral, endogenous, or peptide library origin. FPRL2 in contrast has just recently been assigned its first three agonists of bacterial or peptide library origin. FPR, originally found to have antibacterial function by binding formylated bacterial peptides with high affinity (EC_{50} in the nanomolar range), has now been found to be involved also in inflammation regulation by functioning as a low-affinity receptor for annexin I, which at low concentrations inhibits neutrophil chemotaxis, but at high concentrations activates the neutrophils fully (30).

Our finding that gG-2p20 functions as an agonist for FPR adds a second virus to the known list of pathogens that uses the FPR family receptors. Previously, five HIV-derived peptides have been shown to induce chemotactic activity in neutrophils and monocytes (31). Of these peptides, four bind primarily to FPRL1, while one is FPR binding (but has not been tested for FPRL1 (32)). Hence, our gG-2p20 is the first viral peptide shown to exclusively activate FPR.

Engagement of the FPR by the gG-2p20 showed that this peptide is a potential chemoattractant for human phagocytes in vivo. This feature could contribute to the observed infiltration of neutrophils to the local site(s) of HSV-2 infection (33), where they phagocytose and degrade viral particles (34) and thus contribute to the antiviral defense, as implicated in both experimental animals and humans (13–15). Besides this more obvious reason for the HSV-2 release of a FPR-activating agonist, i.e., to increase the number of phagocytes at the site of infection and thereby increase the tissue damage and thus enabling viral spread and propagation, the FPR (as well as FPRL1) has been shown to be able to down-regulate other chemotactic receptors in vivo. The function of such down-regulation could be a second step in an invasion strategy, prohibiting the attraction of immune competent cells of importance for clearance of the HSV-2 infection. Of course, it could also provide a host protection strategy, limiting the HSV-2 infection. Such hypothesis has been introduced for HIV, in which activation of FPRL1 by HIV envelope-derived peptides down-regulates CCR5 and CXCR4, which are major coreceptors for HIV (21).

The ROS secreted in response to the gG-2p20 peptide were shown to specifically inhibit NK cell cytotoxicity and to induce apoptosis in these cells. This finding corroborates previous studies showing that superoxide radicals down-regulate most NK cell functions (22, 23, 35–39). NK cells rapidly migrate to extravascular sites to attack and destroy altered target cells (40), and they are therefore especially important in controlling HSV infections in which the virus-infected cells otherwise escape CD8$^+$ T cell-mediated destruction as a consequence of the virus-induced down-modulation of MHC class I expression. The number of NK cells at the site of HSV-2 infection tends to drop immediately after HSV reactivation and then increase with time (41). We propose that this reduction in NK cell numbers during acute infection is a result of gG-2p20-induced accumulation and activation of neutrophils and monocytes. It has previously been shown that administration of histamine, which protects NK cells from the suppressive signals induced by phagocytes, prolongs survival of HSV-2-infected mice (but not of mice depleted of NK cells) (42). These data not only confirm that NK cells play an important role during HSV-2 infection, but also indicate that it is indeed ROS that mediate the suppressive effect on NK cell functions in vivo. Thus, the actions of the gG-2p20 peptide on FPR could represent a novel immune evasion mechanism identified for HSV-2. This immune escape might be of importance for the pathogenesis of HSV-2, in particular for the establishment of HSV-2 infection in individuals that are already infected with HSV-1. The acquired immune responses to HSV-1 and HSV-2 are, with the exception of the immune response to the gG-2 protein, highly cross-reactive (43–46). Yet, HSV-2 manages to infect HSV-1-seropositive individuals as efficiently as it infects individuals with no prior HSV exposure (47), perhaps as a consequence of a gG-2-induced ROS-mediated suppression of specific lymphocyte subsets.

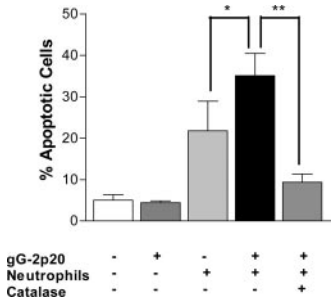

FIGURE 6. Phagocyte-dependent NK cell apoptosis is induced by gG-2p20. NK cell-enriched lymphocytes were monitored for apoptosis after overnight incubation with neutrophils. Stimulation of neutrophils with gG-2p20 significantly increased the percentage of apoptotic lymphocytes, while the peptide itself did not affect lymphocyte viability. The apoptosis-inducing effect of gG-2p20 was clearly mediated by oxygen radicals, as the lymphocytes were protected by the addition of 200 U/ml catalase. Data are expressed as the mean percentage ± SEM of end-stage apoptotic cells (based on the altered scattering properties displayed by apoptotic lymphocytes (see Ref. 29)) ($n = 5$).

FPR is present not only in neutrophils and monocytes, but is expressed also on dendritic cells, astrocytes, hepatocytes, endothelial cells, thyroid cells, adrenal cells, and in the nervous system. The possible interaction of gG-2p20 with FPR at these sites introduces an even more complex view on how HSV-2 may use this secreted protein/peptide at different sites of attack, and could be one mechanism contributing to the devastating consequences of disseminated neonatal HSV-2 infection and also in HSV-2 meningitis. Any gG-2p20-induced effects could, however, only occur at sites of massive local viral replication, as a relatively high dose of gG-2p20 is required for a biological effect (>0.05 μM). Genital blisters represent such a site, where $>10^{10}$ viral particles can be retrieved (our unpublished observation). The liver can also be heavily contaminated and severely damaged, in particular in HSV-2-infected neonates, which sometimes necessitates liver transplantation. However, it still needs to be determined whether native sgG-2 has the same capacity as the sgG-2-derived gG-2p20 peptide to activate chemotaxis and ROS formation in monocytes and neutrophils, and whether proteolytic degradation of sgG-2 could release peptides with such activities.

In summary, we have identified a possible function of the secreted portion of HSV-2 glycoprotein G. A peptide derived from this protein had strong inflammatory properties that influenced the functions of monocytes, neutrophils, and NK cells in vitro. These effects could contribute not only to the inflammatory response to HSV-2, but could also serve as a novel immune escape mechanism used by HSV-2.

Acknowledgments

We thank Marie-Louise Landelius and Maria Hjulström for excellent technical assistance, and Dr. Huamei Fu for help with the transmigration analysis.

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