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IN VITRO DETECTION OF HERPES SIMPLEX VIRUS -1 AND -2 INFECTION WITH IMMUNOSPECIFIC GD^{3+}-CL_6-ENHANCED MAGNETIC RESONANCE IMAGING

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Herpes simplex virus infections are prevalent viral infections in humans. HSVs are also the most common cause of sporadic viral encephalitis (HSE). Magnetic resonance is the imaging method of choice for HSE because it provides the most sensitive method for detecting early lesions. The objective of this study is to set-up and *in vitro* test an experimental contrast agent specific for antigens present on HSV-infected cells, bound with a paramagnetic agent detectable by MR imaging. A selected anti-HSV HrFab was labelled with Alexa Fluor 488, ^{125}I and $Gd^{3+}Cl_6$. In order to assess anti-HSV affinity and specificity, ELISA assays were performed. Vero cells infected with HSV strains were visualized by MRI using anti-HSV HrFab/$Gd^{3+}Cl_6$ complex. Results of the ELISA tests demonstrated that the anti-HSV HrFab labelled with $Gd^{3+}Cl_6$ showed similar affinity for the antigens while the ^{125}I immunoconjugate showed reduced affinity. MRI confirmed high affinity and specificity of antibody for the detection of HSV infections.

Herpes simplex virus (HSV) infections are among the most prevalent viral infections in humans; they are usually responsible for benign recurrent mucocutaneous lesions (1). Two serotypes of HSV are distinguished. HSV type-1 (HSV-1) is usually associated with mouth and eye infections, while HSV type-2 (HSV-2) is usually the cause of genital infections acquired by sexual transmission (2). HSV-2 is the most common cause of genital herpes in the majority of countries where it is responsible for approximately 85% of cases, and is the HSV type involved in 70% of neonatal herpes (3). Neonatal herpes can result from infection with either HSV-1 or HSV-2 (HSV 1-2), the latter being associated with a poorer prognosis (4). Consequences of neonatal HSV infection can be severe. Mortality following multiorgan disease in the absence of therapy is very high (80%), and most infants surviving central nervous system (CNS) disease or disseminated disease are neurologically impaired (35% of HSV-infected infants) (5). HSVs are also the most common cause of sporadic viral encephalitis. In the USA and Europe HSV infection is also the prevalent cause of acute viral encephalitis in immunocompromised adults, due to the emerging pandemic spread of human immunodeficiency virus (HIV). It is difficult to distinguish Herpes simplex encephalitis (HSE) from other forms of encephalitis due to the aspecificity of the symptoms (seizures, lethargy, irritability, tremors, temperature instability, bulging fontanel and pyramidal tract signs).

Before the advent of HSV detection by polymerase chain reaction (PCR) in the cerebrospinal fluid (CSF), the culture of brain tissue obtained by biopsy was

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considered the gold standard for diagnosis of HSE (6). PCR can be successfully used to diagnose HSE between 24-48 hours and 10 days from infection, but sometimes the amplifications of viral nucleic acid may not distinguish the true nature of genomic material (i.e. genomic fragments, latent infection, low-grade persistent infection, active infection) (7). Other procedures to detect viruses in tissue samples include immunofluorescence/immunoperoxidase staining, histopathology and electron microscopy, but all are less sensitive and less specific than virus culture. On the other hand, invasive brain biopsy is rarely performed.

Imaging techniques can be used to exclude focal or diffuse parenchymal pathologies that may mimic HSE symptoms (brain tumours, abscesses, other forms of encephalitis) and to corroborate clinical data evidencing alterations compatible with HSE diagnosis.

Computed tomography (CT) plays a limited role because it can be completely normal in the early stage of disease. At present, magnetic resonance imaging (MRI) is the imaging method of choice for HSE because it provides the most sensitive method for detecting early lesions (8). In several cases, however, MRI findings are not reliable and different pathologic entities may emulate HSE findings, leading to misdiagnosis. The targeting of immunospecific probes and tracers has been demonstrated in the oncologic field in a wide range of neoplastic clones (9-12). In addition, it could allow the visualization of infected tissue otherwise difficult to diagnose.

The aim of our study is to elaborate and to test *in vitro* an experimental contrast agent obtained binding a human recombinant antibody fragment (HrFab) specific for antigens present on the HSV-1 and HSV-2 infected cells to a paramagnetic agent detectable by MRI.

MATERIALS AND METHODS

Cell Lines and Viruses

Vero cells (ATCC CCL-81) and corneal epithelial SIRC cells (ATCC CCL-60) were cultured in Eagle's Minimal Essential Medium (MEM) (Euroclone, Milan, Italy), supplemented with 10% foetal calf serum (Euroclone).

Viruses used in this experiment were HSV-1 Mac Intyre (ATCC VR-539), HSV-2 (ATCC VR-739), and HSV-1 clinical isolate from keratitis.

Labeling Protocols

Anti-HSV HrFab was cloned by phage display from a human bone marrow library of IgG1k, constructed from donor volunteer patients (13). The selected HrFab was named HSV45. HrFab antibody was labelled by using Alexa Fluor 488 monoclonal antibody labeling kit (Invitrogen Corporation) in accordance with the manufacturer's instructions to generate Fab-HSV45/fluor antibody.

HSV45 was also labelled with ^{125}I. HrFab was functionalized with diethylenetriaminepentaacetic acid (DTPA) to allow the covalent binding of ^{125}I. 0.096 nmol $Na^{125}I$ in 0.01 M NaOH were added to 1.44 nmol of HrFab-DTPA anti-HSV1-2. The ratio Fab-DTPA/$Na^{125}I$ was 15:1. The mix was incubated 7 minutes at room temperature, stopped with $NaHSO_3$ and eluted by size-exclusion NAP-5 column. A fraction was controlled with γ-counter (1282 Compugamma). This technique allowed a 70% labeling efficacy.

To label with $Gd^{3+}Cl_6$, equimolar concentrations of Fab-HSV45 and $Gd^{3+}Cl_6$ were used (2 nmol). Fab-HSV45 and $Gd^{3+}Cl_6$ were dissolved in 0.9% NaCl solution and incubated for 12 h at room temperature. The complexes were then dialyzed against deionized water. This technique obtained a 90-95% labeling efficacy. The controls demonstrate that increasing the concentration of Gd for the labeling reduced the affinity of HrFab for its antigen.

ELISA Assays

In order to assess affinity and specificity of Fab-HSV45 and Fab-HSV45/$Gd^{3+}Cl_6$ an ELISA assay was performed. The extracted viral antigen EIA (Ag EIA) of HSV-1 and HSV-2 (Virion Ltd, Rüschlikon - Switzerland) was coated (0.5 μg/ml) on ELISA multiplates and blocked with 10% BSA. 0.05% Tween 20 in PBS was used as washer, and goat anti-human IgG specific HRP conjugated was used as secondary antibody. After washing, the multiplates were stained with BM blue POD substrate (Roche) and spectrophotometrically revealed at 450 nm.

ELISA controls of Fab-HSV45 and Fab-HSV45/$Gd^{3+}Cl_6$ were also performed on i) 2 x 10^6 Vero cells (methanol 50% fixed) infected with: HSV-1 and HSV-2; and on ii) 2 x 10^6 SIRC cells (methanol 50% fixed) infected with HSV-1 clinical isolate. Infected cells were incubated with equimolar concentration of Fab-HSV45/$Gd^{3+}Cl_6$, Gd/DTPA (Magnevist - Schering, Berlin, Germany) and with Fab-HSV45 alone used as control.

Magnetic Resonance Imaging

Vero cells infected with the two HSV strains were

incubated for 1 h with Fab-HSV45/$Gd^{3+}Cl_6$, Gd/DTPA, and with Fab-HSV45 alone used as control. After washing, cell samples were dispersed in Agarose Nusieve (Difco Ltd, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in order to obtain a thick slide to be visualized by MRI. MRI was performed using 1.5-T unit (Magnetom Vision Plus, Siemens Medical Solutions, Erlangen, Germany) with a standard circular polarized head coil. Test vials were firmly positioned parallel to each other and fully submerged in distilled water to avoid susceptibility artefacts from surrounding air. Imaging protocol included coronal and axial T1-weighted spin-echo sequence (TR: 600ms/TE: 15 ms; scan time 45s; FOV: 200 mm x 200 mm, imaging matrix: 256 x 256; averages: 2; slice thickness: 2 mm; pixel size: 0.79 mm x 0.78 mm).

Coronal images of infected cells and controls were qualitatively analyzed by determining any visible contrast agent effect. For quantitative data analysis, the obtained datasets were transferred as DICOM images to an independent console (Aquarius, Terarecon Inc., San Mateo, CA, USA): placing a circular region of interest (RoI) within an area of 20 mm^2 on each vial, the mean signal intensity (SI) +/- Standard Deviation of each test vial and the background noise in frequency encoding direction were recorded and expressed as signal to noise ratio (SNR).

RESULTS

Anti-HSV Human recombinant antibody fragment (HrFab) was cloned by phage display (13) from a human library constructed from a bone marrow donor volunteer patient. For this experimental setting we chose a clone exhibiting a high efficiency of HrFab production and a high antigen affinity for the antigens specifically present only on the membrane of infected cells. The selected HrFab was named HSV45.

The results of the ELISA tests performed using EIA Ag HSV 1-2 demonstrate that both the Fab-HSV45 and Fab-HSV45/$Gd^{3+}Cl_6$ show similar affinity for the antigens while the immunoconjugate containing ^{125}I shows a reduced affinity. The control experiments performed on HrFab-DTPA show that the procedure adopted to bind ^{125}I strongly reduces its binding properties, probably due to structural damages of the antibody (Table I).

An ELISA test was performed using Fab antibodies on SIRC cells infected with HSV-1 clinical isolate strain (deriving from keratitis).

Table I. *ELISA control performed with immunoconjugates on EIA Ag HSV -1 and HSV-2.*

Fab dilution	Fab	Fab-DTPA	Fab-DTPA/^{125}I	Fab-fluor	Fab/$Gd^{3+}Cl_6$
1:2	1.981	1.650	0.623	1.836	1.802
1:20	1.032	0.958	0.412	1.002	0.959
1:40	0.897	0.736	0.312	0.836	0.846
1:80	0.702	0.625	0.120	0.689	0.713
1:160	0.630	0.510	0.061	0.626	0.608
1:320	0.496	0.382	0.063	0.418	0.439
1:640	0.337	0.220	0.064	0.310	0.308
c.c.[a]	0.073	0.070	0.068	0.070	0.067
s.c.[b]	0.048	0.060	0.058	0.052	0.047

Data are the average of four independent measurements. [a]Conjugated control; [b]Substrate control
EIA, extracted viral antigen; Ag, antigen; DTPA, diethylenetriaminepentaacetic acid; fluor, Alexa Fluor 488.

Both Fab-HSV45 and Fab-HSV45/$Gd^{3+}Cl_6$ show similar affinity for EIA Ag HSV 1-2. Fab-DTPA/125I showed a reduced affinity for EIA Ag HSV 1-2. The control experiments performed on HrFab-DTPA show that the procedure adopted to bind ^{125}I strongly reduces its binding properties.

Table II. *ELISA control performed on rabbit HSV-infected corneal epithelial cells (SIRC).*

Virion/cells ratio (MOI)	Fab 1:300	Fab 1:600
MOI 1	0.279 ± 0.009	0.285 ± 0.026
MOI 0.1	0.146 ± 0.009	0.143 ± 0.009
MOI 0.01	0.047 ± 0.014	0.041 ± 0.011
Control[a]	0.044 ± 0.005	0.047 ± 0.005

Data are the average of four independent measurements. [a]Uninfected cells . MOI, multiplicity of infection.

The high affinity of immunoconjugate for the antigenic structure exposed on the surface of infected cells was confirmed by an ELISA test performed by using Fab antibodies on SIRC infected with HSV-1 clinical isolate strain.

Table III. *ELISA control performed on infected Vero cells.*

Virion/cells ratio (MOI)	Fab 1:300	Fab 1:600	Fab/fluor 1:300	Fab/fluor 1:600	Fab/$Gd^{3+}Cl_6$ 1:300	Fab/$Gd^{3+}Cl_6$ 1:600
MOI 1	0.301	0.286	0.288	0.270	0.297	0.300
MOI 0.1	0.150	0.153	0.145	0.146	0.152	0.148
MOI 0.01	0.035	0.038	0.029	0.032	0.024	0.036
Control[a]	0.033	0.035	0.032	0.036	0.029	0.030

Data are the average of four independent measurements. [a]Uninfected cells MOI, multiplicity of infection; fluor, Alexa Fluor 488

The high affinity of both Fab-HSV45/fluor and Fab-HSV45/$Gd^{3+}Cl_6$ to the antigens exposed on the surface of infected cells was confirmed by ELISA tests performed on infected Vero cells used as substrate.

Results are shown in Table II. The high affinity of immunoconjugate for the antigenic structure exposed on the surface of infected cells was confirmed.

Basing on these results, further experiments were performed using only Fab-HSV45/fluor and Fab-HSV45/$Gd^{3+}Cl_6$ antibodies. ELISA tests performed on infected Vero cells used as substrate confirm the high affinity of both immunoconjugates to the antigens exposed on the surface of infected cells (Table III).

In order to validate the specificity of Fab-HSV45/$Gd^{3+}Cl_6$ for HSV infected cells, MRI analysis was performed. As controls of the experiments we used: uninfected Vero cells incubated either with Fab-HSV45/$Gd^{3+}Cl_6$ or with Gd/DTPA, and infected Vero cells incubated with Gd/DTPA (Fig. 1). In all controls the signal intensity was lower than that recorded incubating infected Vero cells with Fab-HSV45/$Gd^{3+}Cl_6$ antibody. These results confirm the high affinity of Fab-HSV45 for HSV infected cells and, on the other hand, demonstrate that the signal intensity of uninfected cells is not enhanced

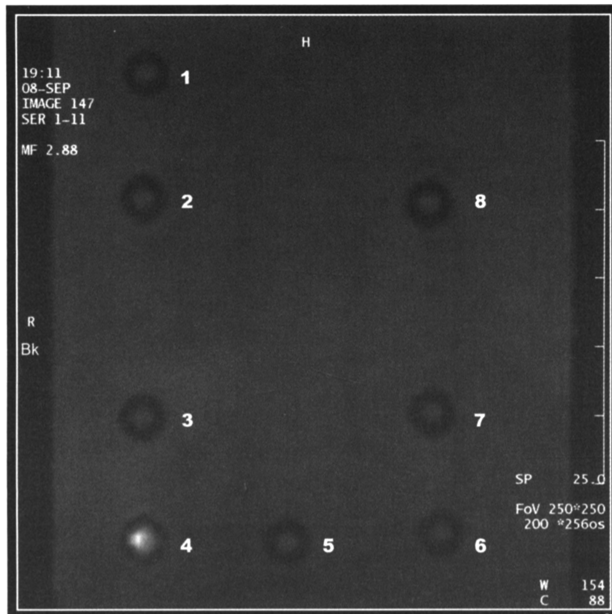

Fig. 1. *Magnetic resonance imaging analysis performed on HSV infected or uninfected Vero cells, embedded in agarose slice. Wells 1-3: agarose slice controls (1, PBS; 2, H_2O; 3, 0.9% NaCl); 4, HSV-infected Vero cells incubated with Fab-HSV45/$Gd^{3+}Cl_6$ antibody; 5: Uninfected Vero cells; 6: Uninfected Vero cells incubated with Gd-DTPA; 7: Infected Vero cells incubated with Gd-DTPA; 8: Uninfected Vero cells incubated with Fab-HSV45/$Gd^{3+}Cl_6$ antibody.*

by Fab-HSV45/$Gd^{3+}Cl_6$ complex after washing, and that unbound gadolinium cannot stably adhere to the membrane of infected cells.

DISCUSSION

HSV 1-2 infection, especially when CNS is involved, can produce severe neurological consequences and, in a relevant percentage of cases, death. The most reliable and non- invasive tools available for diagnosis are PCR and real time PCR (14). Imaging mainly relies on MRI to exclude other parenchymal pathologies and to corroborate laboratory and clinical findings; however, in many cases HSE signs can be unspecific and puzzling. In such cases, conventional imaging could drastically benefit from the use of an immunospecific contrast agent.

The original intuition of an immunospecific contrast agent for MRI dates back to the early 80's: Lauffer first (15), and Renshaw shortly after (16), postulated and tested the production of so-called "paramagnetic proteins", i.e. bovine immunoglobulines and specific human antibodies bound to paramagnetic ligands as gadolinium or magnesium. The technological progress in the field of biology, immunology and medical diagnostic imaging has disclosed the chance to design and produce disease-specific contrast agents to be used in various imaging modalities. As regards MRI, the main target of the industrial and biomedical efforts is represented by the imaging of tumors: immunospecific contrast agents have been tested in a wide range of neoplastic clones, including lymphoma (9), breast cancer, melanoma (10), colorectal carcinoma (11), and esophageal cancer (12). Nevertheless, the extra-oncologic role of MRI immunospecific contrast agents has been merely considered.

Using validated protocols (13), we developed the Fab-HSV45, a recombinant antibody fragment specific for antigens present on the HSV 1-2 infected cells; the affinity and specificity of antibody for EIA Ag of HSV 1-2 were repeatedly tested on ELISA before (Fab-HSV45) and after (Fab-HSV45/ $Gd^{3+}Cl_6$), binding them with an MRI detectable molecule. Results shown in Table I clearly demonstrate that high specificity and sensitivity of the Fab-HSV45 for the antigens is not modified by the presence of ligands with the only exception of the binding to ^{125}I.

In vivo toxicity of Fab and of immunoconjugate (Fab-HSV45/$Gd^{3+}Cl_6$) has been evaluated by Irvin test in the Research Toxicology Center (RTC S.p.A. Pomezia, Italy). Fab was not toxic in mice up to a concentration 1000-fold higher than that used for human use (basing on the quantity injected for diagnostic scintigraphy). Immunoconjugate was not toxic in mice up to a concentration ten-fold higher. The *in vitro* toxicity of Fab and immunoconjugate were also tested: they were both negative. These results obtained the approval of the Ethics Committee of Policlinico Umberto I (Rome, Italy).

The results shown in the present work demonstrate that the combination of the antibody fragment (the immunospecific part of the compound)

with the Gd molecule (the MRI detectable part of the compound) does not affect (in particular does not diminish) the affinity for HSV 1-2 antigens: $Gd^{3+}Cl_6$ is safe and can be used to label HrFab-HSV45 without compromising both its structure and its antigen-specific binding sites. MRI tests confirm the specificity of Fab-HSV45/$Gd^{3+}Cl_6$ for HSV 1-2 infected Vero cells and demonstrate that the molecular structure of the antibody does not alter the paramagnetic effects of Gd: in each case the signal intensity of infected cells that have been incubated with Fab-HSV45/$Gd^{3+}Cl_6$ was higher than that measured for the control samples. Furthermore, a very recent publication has demonstrated that a Gd-DOTA conjugated antibody fragment showed good blood-brain barrier permeability (17).

These *in vitro* findings show the feasibility of immunospecific $Gd^{3+}Cl_6$-enhanced imaging of HSV infection. Additional *in vivo* experimentation are required.

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