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Protective properties of non-nucleoside reverse transcriptase inhibitor (MC1220) incorporated into liposome against intravaginal challenge of Rhesus Macaques with RT-SHIV

Mélanie Caron [a], Guillaume Besson [a], Sonia Lekana-Douki Etenna [a], Armel Mintsa-Ndong [a], Spyridon Mourtas [b], Antonia Radaelli [c], Carlo De Giuli Morghen [c], Roberta Loddo [d], Paolo La Colla [d], Sophia G. Antimisiaris [b,e], Mirdad Kazanji [a,f,*]

[a] Unité de Retrovirologie, Centre International de Recherches Médicales de Franceville, Franceville, BP 769, Gabon
[b] Laboratory of Pharmaceutical Technology, Department of Pharmacy, Université of Patras, Rio, 2050 Patras, Greece
[c] Department of Medical Pharmacology, University of Milan, Milan, Italy
[d] Department of Biomedical Sciences and Technologies, University of Cagliari, Italy
[e] Institute of Chemical Engineering and High-Temperature Processes-FORTH, GR-26500, Patras, Greece
[f] Réseau International des Instituts Pasteur, Institut Pasteur, Paris, France

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ABSTRACT

In the absence of an effective vaccine against HIV, it is urgent to develop an effective alternative such as a microbicide. Single and repeated applications of MC1220 microbicide were evaluated in macaques. First, animals were given a single application of 0.5% or 1.5% MC1220-containing liposomal gel. A second group were treated with 0.5% MC1220 once a day for 4 days. The control groups were treated by liposomal gel alone. Thirty minutes after the last application, animals were challenged with RT-SHIV. In the first protocol, 2 of 4 animals treated by 0.5% of the MC1220 and 2 of 5 treated by 1.5% were protected. In the second protocol, 3 of 5 treated animals were protected and 5 of 5 controls were infected. The RNA viral load at necropsy was significantly lower ($p = 0.05$) in treated-infected animals than in controls. In both protocols, the number of CD4+ T cells was lower at viremia peak in infected than in protected animals.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is the main sexually transmitted infection worldwide, affecting approximately 34 million people (Garcia-Lerma et al., 2008). About 2.5 million people are newly infected each year, and 2.1 million of them die, adding to the 19 million people who have already died from diseases associated with this infection (McGowan, 2006). In the absence of an effective HIV-1 vaccine, the virus continues to spread, with the highest prevalence of infection in developing countries, especially in sub-Saharan Africa and South-East Asia (Klausner et al., 2003). Transmission in Africa is usually heterosexual, and women now account for nearly 50% of the prevalence of HIV/AIDS worldwide (Ambrose et al., 2007) and, unfortunately, few people use condoms to limit the spread of HIV. Therefore, in addition to more effective therapeutic tools, there is an urgent need for cost-effective preventive measures to prevent the spread of HIV/AIDS.

Microbicides are user-friendly, convenient and readily available and would extend the range of means for self-protection against HIV, both in developing and industrialized countries. They limit sexual transmission of the virus (Stone, 2002) by acting on the epithelial barrier of the vaginal and cervical mucosa, which have been implicated in HIV transmission (Galvin and Cohen, 2004). Microbicides must be safe, effective, affordable and acceptable. They should not cause local irritation or epithelial damage, and they should inhibit the virus at its point of entry through the vaginal mucosa, prevent all subsequent steps leading to infection of the host, block viral replication and have a high genetic barrier to resistance (McGowan, 2006; Veazey et al., 2005). Microbicides would appear to be a good preventive method before intercourse (Shattock and Moore, 2003).

Despite more than 20 years of research, however, no microbicide is currently available to provide sterilizing immunity against HIV-1 (Duerr et al., 2006). Of the different types of microbicide, only two

* Corresponding author. Institut Pasteur de Bangui, Bangui, Central African Republic.
E-mail address: mirdad.kazanji@pasteur.fr (M. Kazanji).

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have been tested in phase III trials, PRO2000® and Carraguard® (Abdool Karim, 2010; Skoler-Karpoff et al., 2008) but it is difficult to predict whether these microbicides will have a beneficial or a deleterious effect on HIV transmission (Teleshova et al., 2008). As a proved spermicide tested to prevent against sexually transmitted diseases, the Nonoxynol-9 reduced the susceptibility of gonorrhoea infections otherwise increased the frequency of genital ulcers and vulvitis (Kreiss et al., 1992; Roddy et al., 1998). In clinical trials, microbicides benefit is not deftly evaluated even though a moderate effect was statically monitored. The microbicides efficacy might depend on adherence variability to the trial, independently to the action mechanism of microbicides.

Microbicides that specifically inhibit HIV transmission include monoclonal antibodies against envelope glycoproteins (gp120), attachment receptors and chemokine co-receptors (CCR5 or CXCR4) (Hu et al., 2004; Jiang et al., 2005; Schols, 2004; Veazey et al., 2003); inhibitors of fusion between HIV and its target cells (Derdeyn et al., 2000; Kilby et al., 1998); and reverse transcriptase (RT) inhibitors (Van Herrewege et al., 2004a,b). In the last substance category, several have been already tested in phase II clinical trials as Tenofovir®, UC-781® or TMC-120® (McGowan, 2006); others are tested in preclinical trials (Ambrose et al., 2007). MC1220 is a non-nucleoside RT inhibitor (NNRTI) microbicide of the 3,4-dihydro-2-alkoxy-6-benzyl-4-oxo-pyrimidines (DABOs) series. Pani et al. (2001) demonstrated the ability of DABOs to suppress HIV replication for an entire experimental period of 40 days with no cytotoxicity indication. This property correlates with their ability to tightly bind to the HIV-1 reverse transcriptase. Furthermore, of these series, MC1220 was with the highest *in vitro* potential, showing a "memory effect," i.e. the capability of knocking out HIV replication in freshly infected cells after a 4-h treatment followed by incubation of the extensively washed infected culture in the continuous absence of the drug. We hypothesized that MC1220 could inhibit the intravaginal transmission of HIV by hindering the conversion of viral RNA into DNA, resulting in a reduction to viral integration. In preclinical trials, various *in vitro* models were tested and then, *in vivo*. Veazey *et al.* have demonstrated a dose-dependent protection by monoclonal antibody against vaginal HIV transmission in non-human primates (Veazey et al., 2003). Therefore, rhesus macaques seem well-established *in vivo* models for the HIV-1 transmission (Harouse et al., 2001).

In this study, we evaluated the protective efficacy of MC1220 in a non-human primate model: adult female rhesus macaques. Dose and time effects of MC1220 in preventing the vaginal transmission of SHIV89.6P were evaluated by single and repeated applications. Animals were followed up virologically and immunologically to determine the RNA viral load, the DNA proviral load (in peripheral blood mononuclear cells (PBMCs)), antibody responses, immunological changes (evolution of T-cell subsets) and the viral and proviral load in PBMCs and various lymphoid organs at necropsy.

Results

Irritation effect after intravaginal treatment

The scores for irritation, as measured by the previously described methods (D'Cruz et al., 2003), produced after two doses of MC1220 microbicide incorporated into liposomal gel are summarized in Table 1. Little irritation was seen, with mean scores of 1.08 with 0.1% and 0.56 with 0.5% MC1220. The most irritation was observed in the control group treated by the liposomal gel without microbicide (total score, 1.44), which was significantly different ($p = 0.002$) of the result from the animals group treated by liposomal gel containing 0.5% MC1220 (total score, 0.5). No erythema or edema was detected with 1.5% MC1220 in liposomal gel (data not shown).

Table 1
Mean vaginal irritation scores for monkeys treated with the microbicide MC1220 incorporated in liposome or with the liposomal gel alone (controls).

Vaginal component (range of possible score)	Control liposome alone	MC1220	
		0.1% gel + liposome	0.5% gel + liposome
Erythema	1.02 ± 0.3	0.78 ± 0.36	0.4 ± 0.26
Oedema	0.42 ± 0.32	0.3 ± 0.24	0.16 ± 0.17
Total score	1.44	1.08	0.56

The individual irritation score was assigned on the basis of a semi-quantitative scoring system for inflammation: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = intense. The cumulative scores for erythema and Oedema formation were: < 4 = acceptable, 5–6 = marginal and > 6 = unacceptable

Plasma viral load and PBMC proviral load after challenge

In Protocol 1 (single application, Fig. 1), four of five control animals became infected after challenge, with high plasma viremia (10^7–10^8 copies/ml) and a high proviral load at week 2 after challenge (Fig. 2A). In contrast, no virus was detected in PBMCs of two of the four animals (#R08 and #R09) treated by 0.5% MC1220 (Fig. 2B) and two of the five animals (#R11 and #R13) treated by 1.5% MC1220 (Fig. 2C).

In Protocol 2 (repeated applications), all five control animals became infected after challenge, with high plasma viral load (10^6–10^9 copies per ml) and high proviral load (10^4–10^6 copies per 10^6 PBMCs) (Fig. 2D). RT-SHIV was not detected, however, in the plasma or PBMCs of three of the five animals treated four times by 0.5% MC1220 during the 8 weeks after challenge (Fig. 2E).

Virus isolation from PBMCs of treated and challenged animals

In order to confirm the above observations, we isolated the virus from PBMCs at various times after challenge. As seen in Fig. 3, the results confirmed those obtained for the plasma viral load and the proviral load. RT-SHIV could not be isolated from one control animal in Protocol 1, but all the other animals were positive. In the groups treated by 0.5% and 1.5% MC1220, RT-SHIV was isolated from two of four and three of five animals, respectively (Fig. 3A, B, C). RT-SHIV was isolated from one animal (#R14) at week 3 after challenge but not thereafter (Fig. 3C).

In Protocol 2, RT-SHIV was isolated from all the control animals (Fig. 3D) but from only two of five animals treated four times by 0.5% MC1220 (Fig. 3E). The virus was not found in PBMCs of the remaining three animals (#R21, #R22, #R24) during the 14 weeks of this study (Fig. 3E).

Evaluation of antibody responses after challenge

The antibody response to RT-SHIV was evaluated by ELISA at various times after challenge. All RT-SHIV-infected animals in both protocols developed antibodies to RT-$SHIV_{SIVmac239}$ proteins; however, no antibodies were detected in protected animals, i.e. animals #R08 and #R09 treated by 0.5% MC1220, #R11 and #R13 treated by 1.5% MC1220 in Protocol 1 and animals #R21, #R22 and #R24 treated four times by 0.5% MC1220 in Protocol 2 (online supplementary data 1).

RT-SHIV isolation and proviral load in organs after necropsy

In order to evaluate the efficiency of MC1220 in inducing complete protection, all animals were necropsied 14 weeks after challenge. RT-SHIV was isolated by co-culture from various organs of control group, although animal #R04 appeared to be negative in Protocol 1. No RT-SHIV was found in 50% of animals treated by 0.5% or 1.5% MC1220. Furthermore, in the group treated by 1.5% MC1220, the cell-associated viral load in lymph nodes was lower than in the other groups or undetectable. In Protocol 2, RT-SHIV was detected in all organs of

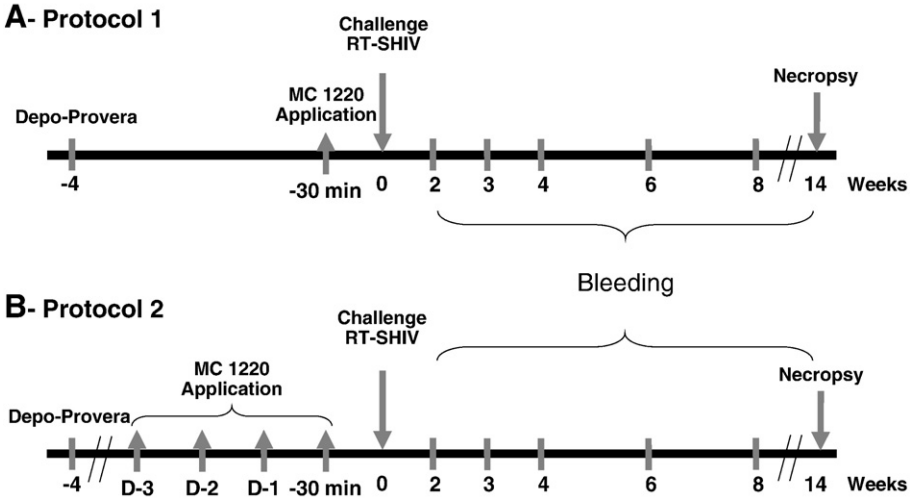

Fig. 1. Treatment protocol. (A) Protocol 1, a single application of 0.5% and 1.5% MC1220 incorporated into liposomal gel (9 treated and 5 control animals). (B) Protocol 2, repeated applications–four times at one day interval before the challenge day–of 0.5% MC1220 incorporated into liposomal gel (5 treated and 5 control animals). Animals were challenged intravaginally, 30 min after the single or last application, with 4.10^5 $TCID_{50}$ of RT-SHIV.

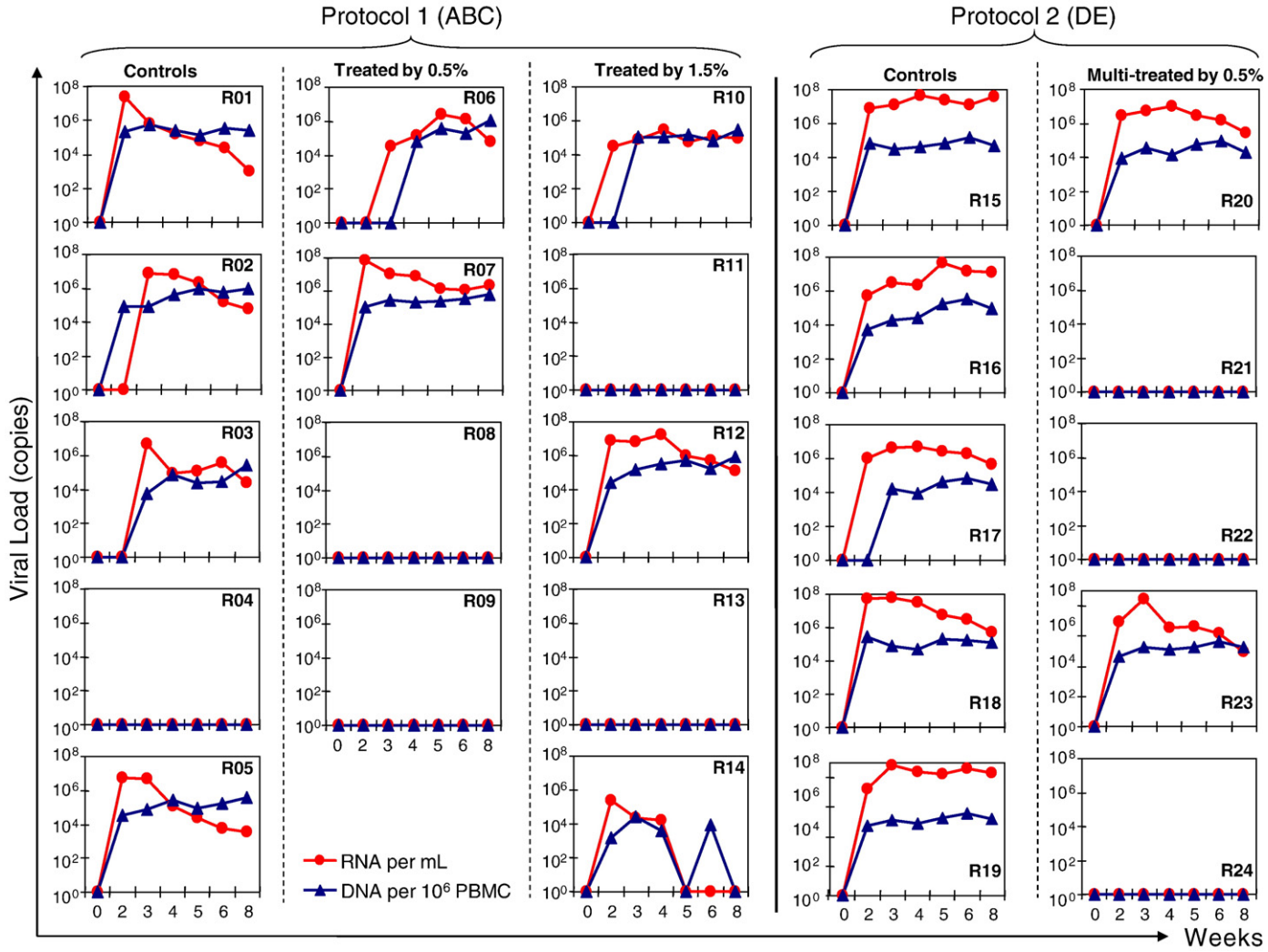

Fig. 2. RT-SHIV plasma viral and PBMC proviral load at various time after challenge. (A, B, C) Protocol 1 (single application): (A) five control animals treated by the liposomal gel alone; (B) four animals treated by 0.5% MC1220; (C) five animals treated by 1.5% MC1220. (D, E) Protocol 2 (repeated applications): (D) five control animals treated by the liposomal gel alone; (E) five animals treated four times by 0.5% MC1220. Plasma viral load (RNA) is in red and PBMC proviral load is in blue (DNA).

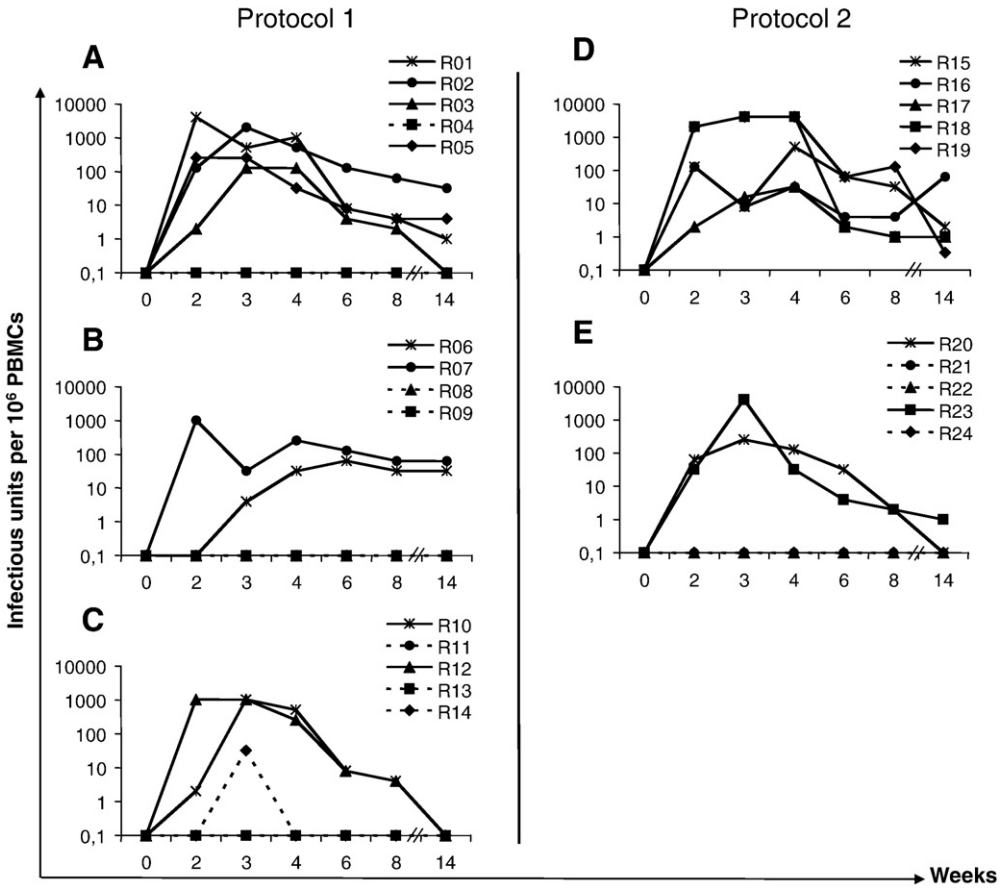

Fig. 3. Cell-associated viral load from PBMCs over time after intravaginal challenge with RT-SHIV. (A, B, C) Protocol 1 (single application): (A) five control animals treated by the liposomal gel alone; (B) four animals treated by 0.5% MC1220; (C) five animals treated by 1.5% MC1220. (D, E) Protocol 2 (repeated applications): (D) five control animals treated by the liposomal gel; (E) five animals treated four times by 0.5% MC1220.

control group but in only two of the five animals treated four times by 0.5% MC1220 (online supplementary data 2).

The presence of RT-SHIV in organs was also evaluated by RT-PCR and real-time PCR. As shown in Table 2, all the control animals were positive, except animal #R04 in Protocol 1. Organs from two animals treated by 0.5% MC1220 (#R08 and #R09) and two animals treated by 1.5% MC1220 (#R11 and #R12) were negative. In Protocol 2, the organs from control animals were all positive, but no RT-SHIV was detected in three animals (#R21, #R22, #R24) treated four times by 0.5% MC1220.

The mean viral load of all organs was compared for control and treated but infected animals. As shown in Fig. 4B, the RNA viral load at necropsy (week 14) was significantly lower ($p = 0.05$) in the infected animals treated in Protocol 2 than in control group. No significant difference was found in Protocol 1 (Fig. 4A).

Evaluation of T-cell subsets in PBMCs and organs at necropsy

T-cell subsets were evaluated before and during the 14 weeks after challenge in controls, treated–infected and protected animals and then, at necropsy in several lymphatic organs (online supplementary data 3). The number of CD4+ T-cells had decreased significantly in control and treated–infected animals at the week 4, but not in the protected group. The lowest number of CD4+ T-cells was found at the week 6 in controls and treated–infected animals, while the number of CD4+ T-cells was significantly higher in the protected animals than in other groups.

The evolution of CD8+ T-cells during the study was evaluated in the same groups. No significant difference was found in the number of CD8+ T-cells in PBMCs; however, the number was significantly higher in control animals than in treated–infected and protected animals.

At necropsy, no significant difference between groups was found in the percentages of various T-cell subsets in the organs. Nevertheless, the percentage of effector memory T-cells in CD8+ T-cells in the axillary, inguinal and iliac lymph nodes of the protected animals was significantly lower than in the other infected groups (data not shown).

Discussion

In this study, we demonstrated that the MC1220 microbicide is able to induced partial protection in treated animals compared to the controls. Seven out of 14 animals that received the microbicide were uninfected after challenge, compared to 1 out of the 10 controls ($p < 0.05$). In Protocol 1, no significant difference was found in relation to the dose used. However, in the group treated with 1.5%, the cell-associated viral load in the lymph nodes was lower than in monkeys treated with 0.5%. When repeated applications of the microbicide were given, three of the five treated animals were protected, and the two infected animals had a lower plasma viral load than the control group. Our data demonstrated that treatment with MC1220 is also able to reduce the virus load in treated infected animals.

We also showed that the number of CD4+ T-cells decreased significantly in both the control group and in treated but infected animals 6 weeks after intravaginal challenge. In protected animals treated by single or repeated applications, however, the number of CD4+ T-cells was stable, showing that MC1220 induced protection.

Table 2
RT-SHIV plasma viral load and proviral load in PBMCs and various organs after necropsy.

	Control Group					0.5% MC1220 treatment				1.5% MC1220 treatment				
Variable	R01	R02	R03	R04	R05	R06	R07	R08	R09	R10	R11	R12	R13	R14
(A) Monkeys treated in protocol 1, with a single application of the microbicide														
Viral load[a]														
Plasma	2.1×10^4	1.2×10^5	$<10^2$	–	9.5×10^4	1.6×10^6	2.2×10^6	–	–	1.2×10^4	–	1.6×10^5	$-<10^2$	
Proviral load[b]														
PBMCs	3.3×10^5	8.3×10^4	4.5×10^4	–	5.4×10^4	3.8×10^5	2.5×10^6	–	–	1.9×10^5	–	3.0×10^6	–	5.8×10^4
Spleen	1.6×10^4	1.1×10^6	1.8×10^5	–	2.2×10^5	5.7×10^5	9.4×10^5	–	–	6.1×104	–	9.8×105	–	4.9×103
Lymph nodes														
Axillary	4.5×10^5	2.1×10^6	4.2×10^4	–	4.7×10^4	1.5×10^5	1.9×10^6	–	–	1.2×10^5	–	6.9×10^5	–	2.3×10^4
Mesenteric	7.7×10^5	3.1×10^6	4.0×10^5	–	1.7×10^5	3.7×10^5	1.3×10^6	–	–	7.1×10^5	–	1.8×10^6	–	3.2×10^4
Ilialic	1.6×10^6	1.2×10^6	3.7×10^4	–	1.8×10^5	6.1×10^5	2.3×10^6	–	–	5.9×10^5	–	3.9×10^6	–	6.9×10^3
Inguinal	3.2×10^5	3.3×10^6	2.7×10^4	–	3.2×10^3	2.5×10^5	4.7×10^5	–	–	4.3×10^4	–	4.9×10^6	–	1.6×10^4

	Control Group					4×0.5%MC1220 treatment				
Variable	R15	R16	R17	R18	R19	R20	R21	R22	R23	R24
(B) Monkeys treated in protocol 2, with multiple applications of the microbicide										
Viral load[a]										
Plasma	1.6×10^7	1.4×10^7	1.1×10^6	3.8×10^6	1.9×10^6	3.0×10^5	–	–	9.4×10^5	–
Proviral load[b]										
PBMCs	2.4×10^6	1.6×10^6	1.1×10^6	7.2×10^5	4.1×10^6	5.0×10^6	–	–	1.6×10^7	–
Spleen	6.5×10^6	5.1×10^6	2.6×10^6	5.7×10^6	2.2×10^6	2.6×10^6	–	–	2.0×10^7	–
Lymph nodes										
Axillary	6.6×10^6	5.2×10^6	7.5×10^5	3.3×10^6	2.3×10^6	4.2×10^6	–	–	1.9×10^7	–
Mesenteric	4.8×10^6	4.7×10^6	2.2×10^6	4.5×10^6	7.9×10^5	1.3×10^6	–	–	2.1×10^7	–
Ilialic	4.1×10^6	3.8×10^6	1.6×10^6	4.8×10^6	2.3×10^6	2.1×10^6	–	–	2.1×10^7	–
Inguinal	3.4×10^6	1.3×10^6	2.7×10^7	2.5×10^6	1.1×10^6	1.8×10^7	–	–	1.9×10^7	–

[a] RNA copies/mL.
[b] Copies/μg of DNA.

Furthermore, no RT-SHIV was found with various techniques for virus detection in any of the protected animals. These results confirm that MC1220 is effective in inducing complete protection after challenge. One limitation of our study, however, is the small number of macaques in each group, and further experimental studies are needed to confirm our observations.

Research on microbicides is evolving rapidly. It is estimated that 60–80 products are currently in development, most of which are in preclinical evaluation and have already been evaluated *in vitro*. Many candidates, involving various routes, formulations and combinations, are being tested, although it is critical that they do not have to be used in a coitally dependent fashion (McGowan, 2006). NNRTIs may be suitable as they inhibit RT and thus suppress the conversion of viral RNA into DNA before integration into the host genome, resulting in large reductions in viral replication. Two main types of microbicides are being studied: those for oral therapy and topical agents. Several topical products are under clinical development at a reasonable production cost, and many studies with animal models suggest that they are effective. A possible disadvantage, however, is that microbicides that are RT inhibitors might result in antiretroviral resistance if patients use the products for a long time or at a low repeated dose.

Three RT inhibitor microbicides are under clinical assessment: Tenofovir®, UC-781® and TMC-120®. Tenofovir has been evaluated in non-human primates, with 56% efficacy after either cervical or rectal challenge (Cranage et al., 2008). UC-781® is also a rectal and vaginal microbicide but has reduced activity against NNRTI-resistant HIV-1 (Hossain and Parniak, 2006). TMC-120® had high activity against wild-type and mutant HIV (D'Cruz and Uckun, 2006). Attacking

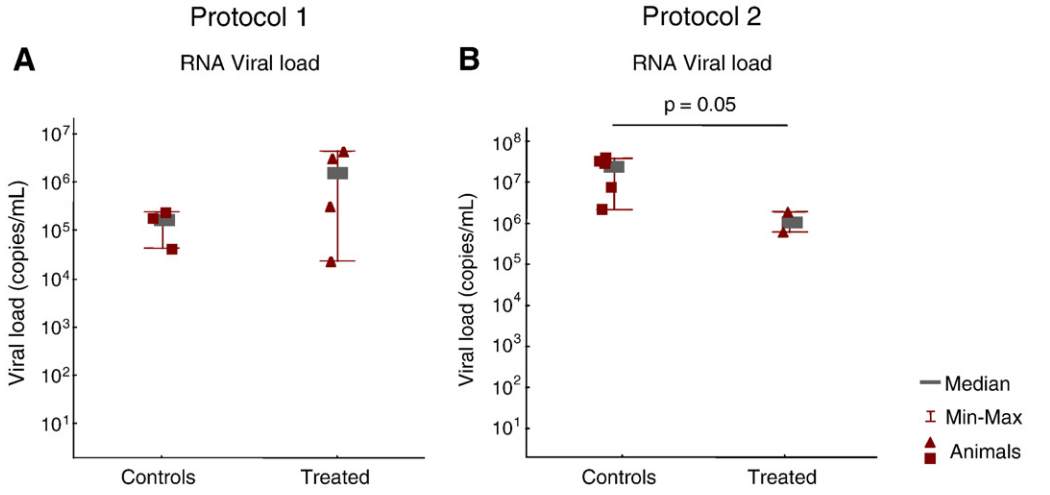

Fig. 4. Plasma viral load at necropsy (14 weeks after challenge) in control and treated but infected animals after challenge. (A) Protocol 1 (single application), with no difference between control and treated animals. (B) Protocol 2 (repeated applications), with a significantly lower plasma viral load ($p=0.05$) in treated but infected than in control animals.

multiple targets could help combat HIV-1 sequence diversity and minimize transmission of variants resistant to any single inhibitor (Veazey et al., 2005). If a topical RT inhibitor like MC1220 results in virus replication, additional inhibition could be provided by other inhibitors, targeting viral entry or enhancing defence. Some such drugs have been developed, such as PRO-2000®, which interferes with the binding of HIV-1 to the CD4 receptor, and PSC-RANTES, which targets specific host cell co-receptors, such as CCR5 (Lederman et al., 2004); however, hepatotoxicity has been seen with some CCR5 antagonists. Furthermore, MC1220 is a NNRTI that shows a 'memory effect', i.e. the ability to knock out HIV replication in heavily infected cells and in dendritic cells and PBMCs *ex vivo* infected with HIV-1 clinical isolates, MC1220 inhibits the different HIV-1 clades with potencies comparable (A, B, C, D, F1, CRF02, NNRTI resistant C) or superior (G, CRF01) to those of UC-781 and TMC-120 (Pani et al., 2001).

As the bioavailability of topical drugs is very low, various approaches have been taken to increase it (Loftsson and Masson, 2001). Use of an innocuous chemical (fatty acids, alcohols, amines) or physical (ultrasound, iontophoresis …) means could enhance and facilitate diffusion of the drug through the barrier or affect its permeability. In our study, the microbicide MC1220 was incorporated into a complex liposomal gel which allows high amount of drug loading in a small volume of formulation.

Unexpectedly, the highest dose used provided only partial protection in Protocol 1. A possible reason for this incomplete dose–response effect could be the low aqueous solubility of MC1220 (1–3.5 ppm, depending on the pH of the solution, with 2–2.5 ppm at pH 5.0). Consequently, the amount of bioavailable drug on the vaginal mucosa did not increase proportionally to the increase in the drug concentration in the formulation, as demonstrated by the similar release rates of MC1220 from the 0.5% and 1.5% liposomal formulations. A strategy to tackle this problem has been identified, and it is hoped that it will improve the formulation and, subsequently, the performance of MC1220. A new approach, therefore, by identifying alternative solutes for MC1220, would increase its solubility in aqueous media; without causing vaginal irritation, and acting as a carrier that would improve the release of the drug from the formulation following its vaginal application. A second approach to increase the protection provided by MC1220 would be to use the active enantiomer of the drug instead of its racemic mixture that is used herein. Theoretically this later approach should result in 2 times whether increase of efficacy.

Münch et al. recently identified a 20-amino acid virus-inhibitory peptide that efficiently blocks HIV-1 entry by interacting with the gp41 fusion peptide (Munch et al., 2007). This peptide might also be included in combination with MC1220 to prevent vaginal transmission of HIV-1.

In conclusion, we have shown that the microbicide MC1220 can induce partial protection after intravaginal challenge of rhesus macaques. We suggest approaches to improving the efficacy of the product, by using different formulation strategies and/or by using several microbicides targeting different sites of virus propagation, like entry inhibitors, adhesion inhibitors and other replication inhibitors. The challenge is to find sufficient financial resources for accelerating microbicide development. First-generation products have shown their limits; effectiveness trials could now improve protection against the main types of HIV, especially with synergetic microbicides like MC1220.

Material and methods

MC1220 formulations in liposomal gel

MC1220 liposomes were prepared using Hydrogenated Egg PhosphatidylCholine (H-PC) purchased by Lipoid (Germany) and Cholesterol (Sigma-Aldrich, France) at 2/1 (mol/mol). The liposomes were prepared in citrate buffer pH 5.0 which contained 0.2% (w/v) sodium benzoate as preservative.

After evaluating different techniques of liposome preparation in terms of encapsulation efficiency, a modified dried-rehydrated vesicle technique with high liposome loading capacity of MC1220, was developed (Antimisiaris S.G. et al., unpublished results). Non-entrapped solid drug was separated of liposomes by sucrose gradient centrifugation in swing-out bucket tubes, at 15.000 rpm for 30 min. Then, the liposomal dispersions stay at a temperature above the lipid transition temperature during 1–2 h for annealing structural defects of the lipid membrane.

Gel preparation and rheological property adjustment

For the rheological property adjustment of the liposomal formulations, we considered a mixture of Carpobol and Hydroxypropylcellulose polymers which has been previously demonstrated to form gels with good characteristics for vaginal delivery (Mourtas et al., 2007). The ideal system would be easy to apply and well distributed to coat (and thus, protect) the whole surface of the vaginal epithelium. After application however, the gel has to stay in place under the low stress conditions applying due to physiological movements of the vaginal area, and this translates to higher viscosity under low stress conditions. Consequently, two polymers (Natrosol® and Carbopol (Stolte-Leeb et al., 2008)) were used for adjustment of the viscosity and the rheological properties of the gels. Then, Glycerol was added in all gels in order to prevent dehydration. The final composition of the liposomal gel used in the *in vivo* experiments is presented in Table 3.

After preparation, gel formulations could be safely stored at 4 °C until used. Lipid concentration of liposomal dispersions was measured by the Stewart colorimetric assay (Stewart, 1980), and final drug concentration in the gels was measured by a validated HPLC technique. Exact lipid and drug concentrations of the various gels utilized are presented in Table 4.

Animals

Twenty-four healthy, cycling adult female rhesus macaques (*Macaca mulatta*) aged 3.7–5.3 years were housed individually in cages at the primate center of the CIRMF, according to the European and the United States National Institutes of Health guidelines for animal care. All the protocols and procedures were approved by the Ethical Committee of Ile-de-France for animal experimentation and by the Gabonese ethics committee for animal experimentation and registered under No. 06-005. The primate center has three veterinarians specialized in primates, comprising an ethologist, a primatologist and an ecologist. All experiments were conducted under their supervision.

The macaques were virologically and serologically negative for Ebola, αHSV, SRV, SIV and STLV. One month prior to virus inoculation, the animals were treated by a single 30-mg intramuscular injection of depo-medroxyprogesterone acetate (Depo-Provera, Pfizer, France) to synchronize their menstrual cycles and to thin the vaginal mucosa, thus increasing their susceptibility to vaginal transmission of virus (Marx et al., 1996). Before inoculation, each macaque was sedated

Table 3
Gelling agents and amounts added in a 20 mL dispersion of MC1220 loaded liposomes (with 40 mg/mL lipid concentration) for rheological property adjustment.

Type of agent	Amount (g) added in 20 ml liposome dispersion
Natrosol 250 HX	0.30
Carbopol 974 P NF	0.08
Glycerol	0.2

Table 4
The gel analysis by Stewrt assay (for lipid measurement) and by HPLC (for MC1220 measurement). The exact amount of MC-1220 incorporated in the different gel types.

Formulation and batch number	Lipid [in liposomal form] (mg/ml) H-PC/Chol (lipoid E PC 3)	MC-1220 concentration (ppm)
Liposome blank	42.60	–
MC-1220 0.5% liposomal	42.57	5419 ± 239
MC-1220 1.5% liposomal	42.62	16559 ± 465

with ketamine (10 mg/kg intramuscularly; Imalgene 1000, Merial, France).

Evaluation of the irritation effect after microbicide treatment

Before the initiation of microbicide treatment, vaginal irritation was evaluated in 15 female macaques in several subgroups receiving 2 ml of various formulations via a urethral catheter to ensure that the total amount of microbicide was introduced to the vagina. The method used for the evaluation of the vaginal irritation effect was previously described by D'Cruz *et al.* (D'Cruz et al., 2003). Animals were maintained with the pelvis in a high position for 30 min to ensure complete gel penetration. Groups of three macaques received 0.5% MC1220 in liposomal gel, 0.1% MC1220 in liposomal gel, or 0.1% MC1220 without liposomal gel for 4 consecutive days. Two subgroups of three animals were given liposome alone or gel alone. Body weights and temperature were obtained before each vaginal application.

The individual irritation score was assigned on the basis of a semi-quantitative scoring system for inflammation: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = intense. The cumulative scores for erythema and edema formation were: < 4 = acceptable, 5–6 = marginal and > 6 = unacceptable (D'Cruz et al., 2003).

Microbicide treatment and virus challenge

The two protocols used are illustrated in Fig. 1. In Protocol 1 (single application), five animals received liposomal gel alone (controls), four received 0.5% MC1220 in liposomal gel, and five received 1.5% MC1220 in liposomal gel. In Protocol 2 (repeated applications), five animals were treated by liposomal gel containing 0.5% MC1220 on days −3, −2, −1 and 0 before RT-SHIV challenge, and five control animals received the liposomal gel alone at the same times. In both protocols, 30 min after the last application of the microbicide, the animals were infected intravaginally with 4×10^5 $TCID_{50}$ of RT-SHIV (an SIV/HIV hybrid virus in which only the reverse transcriptase of SIVmac239 was replaced by that of HIV-189.6P) in seminal human fluid (Uberla et al., 1995) and were followed-up for 10 weeks.

Blood samples were taken every 2 weeks, and PBMCs were separated by centrifugation in lymphocyte separation media. The animals were sacrificed 14 weeks after infection, and tissue samples were collected from the spleen and the mesenteric, axillary, inguinal and iliac lymph nodes.

Determination of plasma RT-SHIV load

After challenge, the presence of RT-SHIV in the plasma was evaluated by RT-PCR. RNA was extracted from 200 µl of plasma with the QIAamp® Viral RNA mini kit (Qiagen, France) and eluted in 60 µl of elution buffer according to the manufacturer's instructions. The viral load was detected by real-time RT-PCR as previously described (Hofmann-Lehmann et al., 2002) with the Quantitect RT-PCR Probe PCR one-step kit (Qiagen, France) on an iCycler iQ5, with primers at a final concentration of 450 nmol/l and with probes at a concentration of 500 nmol/l. Viral RNA was quantified by comparison with a standard curve of log dilutions of cDNA standards (from RT-SHIV) ranging 10^2 to 10^8 copies. Samples (10 µl of RNA per reaction) were run in duplicate. Primers and TaqMan probes were previously described (Hofmann-Lehmann et al., 2002).

Detection of RT-SHIV proviral load

Genomic DNA was isolated from 140 µl of PBMCs with the QIAamp® DNA mini kit (Qiagen, France) and eluted in 200 µl of elution buffer according to the manufacturer's instructions. Proviral DNA was detected by real-time PCR in the same material (except for the PCR reagent, which was Quantitect PCR Probe from Qiagen) with the primers and probe described above. The cycling conditions were as follows: 95 °C for 10 min, followed by 50 cycles at 95 °C for 10 s and 60 °C for 60 s. Viral DNA was quantified by comparison with a standard curve of log dilutions of DNA standards, ranging from 4 to 4×10^6 copies. Samples (5 µl per reaction) were run in duplicate. To determine the mean number of cells that contained proviral DNA, we used parallel amplification of albumin. The cycling conditions were identical to those detailed above. We used the primers *AlbF* 5'-GCT GTC ATC TCT TGT GGG CTG T-3' and *AlbR* 5'-ACT CAT GGG AGC TGC TGG TTC-3' (final concentration, 500 nmol/l each) and the probe *AlbT* 5'-6FAM-CCT GTC ATG CCC ACA CAA ATC TCT CC-TAMRA-3' (final concentration, 300 nmol/l).

Cell-associated virus load

The cell-associated virus load was determined in a limiting dilution co-culture assay with C8166 cells and mononuclear cells from blood and lymphoid organs (spleen and mesenteric, axillary, inguinal and iliac lymph nodes) as described previously (Stahl-Hennig et al., 1996). The cell-associated virus load was expressed as the number of infectious units per 10^6 PBMCs after 2 weeks of co-culture in the weeks 2, 3, 4, 6 and 8 after challenge and the week of necropsy. Plasma and PBMCs were separated by centrifugation on Ficoll density gradients at 1000g for 15 min in Leucosep separation tubes (Greiner Bio-One, Germany). Mononuclear cells were obtained from lymphoid organs by passing the crushed organs through a 40 µm nylon BD Falcon Cell Strainer (BD Biosciences, Belgium). The PBMCs were also subjected to flow cytometry and quantitative real-time PCR analysis.

Virus-specific antibody responses

Antibodies to RT-$SHIV_{SIVmac239}$ were assessed with Genscreen® HIV-1/2 version 2 (Biorad, France) according to the manufacturer's instructions. The plasma samples, diluted 3:4, were tested at the weeks 1, 2, 4, 6, 8, 10 after challenge and the week of necropsy. The antibody responses were determined at optical densities of 450 and 620 nm. The serological data were expressed as optical density/cut-off value, and samples were considered positive at > 1.

Lymphocyte phenotype analysis

To identify virus-specific responses, 30 µl of whole blood were incubated with 6–12 µl of different combinations of monoclonal antibodies mixed at room temperature for 15 min in the dark: for CD4 cells, CD45RA $FITC_{(5H9)}$, CD29 $PE_{(MAR4)}$, CD4 $PerCP_{(L200)}$ and CD3 $APC_{(SP34-2)}$; for CD8 cells, CD45RA $FITC_{(5H9)}$, CD29 $PE_{(MAR4)}$, CD8 $PerCP_{(SK1)}$ and CD3 $APC_{(SP34-2)}$; for effector and central memory CD4 cells, CD95 $FITC_{(DX2)}$, CD28 $PE_{(L293)}$, CD4 $PerCP_{(L200)}$ and CD3 $APC_{(SP34-2)}$; and for effector and central memory CD8 cells, CD95 $FITC_{(DX2)}$, CD28 $PE_{(L293)}$, CD8 $PerCP_{(SK1)}$ and CD3 $APC_{(SP34-2)}$. After addition of 750 µl of lysing solution (Becton Dickinson, Lincoln Park, New York, USA), incubation proceeded for 5 min at room temperature in the dark. After centrifugation at 1800 rpm and elimination of the supernatant, cell fix was added and flow cytometry was performed

on a FACsort with Flowjo software, 10,000 events being analysed per sample.

Statistical analysis

For comparison between groups, Mann–Whitney *U* tests were performed. Correlations between different sets of data for the same group were analyzed with either the standard Pearson correlation coefficient or the Spearman's rank correlation test. Significance was assessed at $p<0.05$. All analyses were performed with Statistica software v7.1 (StatSoft France, www.statsoft.fr).

Competing interests

None.

Author contributions

Mirdad Kazanji and Bettina Sallé conceived and designed the experiments; Mélanie Caron, Guillaume Besson, Sonia Lekana-Douki Etenna, Armel Mintsa-Ndong, Spyridon Mourtas and Sophia G. Antimisiaris performed the experiments; Mélanie Caron, Guillaume Besson and Mirdad Kazanji analysed the data and wrote the paper. Sophia G. Antimisiaris, Antonia Radaelli, Carlo De Giuli Morghen, Roberta Loddo and Paolo La Colla contributed reagents and materials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.06.008.

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