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Mismatched dsRNA (Ampligen) induces protection against genomic variants of the human immunodeficiency virus type 1 (HIV-1) in a multiplicity of target cells

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Summary

Mismatched double-stranded RNA of the form $r(l)_n \cdot r(C_{12}\text{-}U)_n$ (Ampligen) has been shown to be active against human immunodeficiency virus type 1 (HIV-1) using CEM and C3 cells as targets for infection by the highly similar HIV-1 isolates HTLV-III_B and LAV (Montefiori, D.C. and Mitchell, W.M., 1987, Proc. Natl. Acad. Sci. U.S.A., 84, 2985–2989). The scope of Ampligen's anti-HIV-1 activity was examined in this study using the genetically divergent HIV-1 isolate HTLV-III_{RF}, two additional target T-cell lines, H9 and MT-2, and a monocyte/macrophage cell line, U937. As judged by indirect immunofluorescence, reverse transcriptase activity and vital dye uptake, Ampligen was active against HTLV-III_{RF} in H9, MT-2, C3 and U937 cells in addition to being active against HTLV-III_B in U937 cells. A minimum of 1 h preincubation of cells (MT-2) with Ampligen was required for maximum activity. These results suggest that Ampligen's potential clinical efficacy may not be limited by either the highly variable nature or host cell range of HIV-1.

dsRNA, mismatched; HIV-1; AIDS

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Introduction

Human immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS), selectively infects and destroys the host's helper subset of T-lymphocytes, thereby giving rise to severe opportunistic infections and certain neoplasias (Klatzmann et al., 1984; Wong-Staal and Gallo, 1985). Monocytes have also been identified as a major target for HIV-1 infection and may be responsible for the frequent acute encephalopathy associated with AIDS (Koenig et al., 1986). A comparison of various HIV-1 isolates has revealed a high degree of genomic diversity. Whereas isolates such as HTLV-III_B and and LAV differ by less than 2%, the Haitian isolate HTLV-III_{RF} shows much greater divergence, approaching 19% in the envelope gene (Hahn et al., 1985). The most variable region of the HIV-1 genome is the envelope (*env*) gene and variability in this region is thought to be a mechanism for escaping the host's immune response (Hahn et al., 1985). However, this variation as well as variation that occurs throughout the genome could reflect a potential for differential efficacies of antiviral drugs against the many variants of HIV-1; therefore, anti-HIV-1 drug testing should include diverse HIV-1 isolates. In addition, potentially useful anti-HIV-1 drugs should be screened for activity in monocytes as well as T helper lymphocytes since both lineages serve as major targets for HIV-1 and play a role in the pathogenesis of HIV-1 infection in vivo.

Double-stranded RNAs (dsRNAs) are potent biological response modifiers having pleiotropic activities which include induction of the interferons (IFNs), activation of certain IFN-induced enzymes such as 2-5A synthetase, augmentation of natural killer and monocyte activities, and B and T cell mitogenic activities (Carter and De Clercq, 1974; Hearl and Johnston, 1986; Hubbell et al., 1985). These immunomodulatory and antiviral properties make dsRNAs prime candidate drugs for the treatment of AIDS. Indeed, synthetic mismatched dsRNA of the form $r(I)_n \cdot r(C_{12}\text{-}U)_n$ (Ampligen) was shown to have anti-HIV-1 activity in vitro against three similar isolates (LAV and two variants of HTLV-III_B) and in two T-cell lines (CEM and C3) (Montefiori and Mitchell, 1987b). Furthermore, Ampligen and azidothymidine (AZT) acted synergistically against HIV-1 in similar infection assays (Mitchell et al., 1987). In a phase 1/phase 2, 'open' clinical trial, Ampligen improved the clinical status of ten patients with AIDS-related complex (ARC), lymphadenopathy syndrome (LAS) or AIDS while producing only rare, mild side effects (Carter et al., 1987). These results have led to a larger, double-blind, placebo-controlled study with Ampligen, currently in progress, for the treatment of ARC and LAS.

In a continuing effort to characterize the full, potential efficacy of Ampligen in the treatment of HIV-1 infection, we have extended our previous investigations to include the highly divergent HIV-1 isolate HTLV-III_{RF} (Shaw et al., 1984) as well as three additional target cell lines, including the T-lymphoblastoid cell line H9 (Popovic et al., 1984b), the HTLV-I-transformed T cell line MT-2 (Haertle et al., 1988), and the monocyte/macrophage cell line U937 (Sundstrom and Nilsson, 1976). We present the results of these studies in this report.

Materials and Methods

Cells and viruses

Cultures of H9, MT-2, U937, H9/HTLV-III_B, H9/HTLV-III_{RF}, U937/HTLV-III_B and the HTLV-II-transformed T cell line C3 (Montefiori and Mitchell, 1986, 1987a; Popovic et al., 1984a) were grown and maintained at 37°C in RPMI-1640 containing 16% heat-inactivated fetal bovine serum and 50 μg gentamicin/ml. Preparations of HTLV-III_B and HTLV-III_{RF} were obtained from conditioned H9/HTLV-III_B, U937/HTLV-III_B or H9/HTLV-III_{RF} culture fluids. These conditioned fluids were clarified of cells by low speed centrifugation and 0.45 μm filtration. Media were replaced and cell densities reduced every two days to allow continual, exponential cell multiplication.

In vitro infection assays

Infection assays were performed in either 25 cm^2 flasks or 96-well microtiter plates. For infection assays in flask cultures, target cells were challenged with HTLV-III_B or HTLV-III_{RF} at a multiplicity of infection (m.o.i) of 1–5. Virus was allowed to adsorb for 3 h and then was removed by washing with RPMI-1640. Cultures were then incubated in 20 ml of fresh growth medium with or without Ampligen, respectively, as before. Media were replaced with fresh growth medium with or without Ampligen as before and cell densities equally reduced every two days. Samples for indirect immunofluorescence (IIF), reverse transcriptase (RT) activity and vital dye uptake were obtained at these times. IIF was performed microscopically on air-dried, 1:1 acetone:methanol-fixed slides using human anti-HIV-1 p24 serum as described (Montefiori and Mitchell, 1986). RT activity in culture fluids was determined a described by Poiesz et al. (1980) using poly(A)·$(dT)_{15}$ as template primer and 25 μCi [*methyl*-^{3}H]dTTP (80.1 Ci/mmol) per reaction. Vital dye uptake of Finter's neutral red was used to calculate cytopathic effect (Montefiori and Mitchell, 1987b).

Microtiter infection assays in 96-well plates were performed essentially as described (Montefiori et al., 1988) Briefly, MT-2 cells were seeded at a density of 2×10^5 cells/0.2 ml/well and challenged with HIV-1 at an m.o.i. of 1–5 (50 μl of conditioned H9/HTLV-III_B culture fluid containing $2-10 \times 10^5$ infectious particles per well). Plates were incubated in modular incubator chambers flushed with 5% CO_2 in air and assayed for cytopathic effect after four days. Cytopathic effect was quantitated by vital dye (neutral red) uptake of poly-L-lysine adherent cells. Percent protection is defined by the equation:

$$\frac{\text{(Test value minus virus control)}}{\text{(Cell control minus virus control)}} \times 100$$

where test value is the mean A_{540} of 3 samples containing effectors, virus and cells; cell control is the mean A_{540} of 8 wells containing only MT-2 cells, and virus control is the mean A_{540} of 8 wells containing cells and virus without effectors. Infectious viral titers were determined from 50% tissue culture infectious dose ($TCID_{50}$)

values obtained by endpoint microtitration on MT-2 cells in 96-well plates as described (Montefiori et al., 1988)

Results

The antiviral activity of Ampligen against the HIV-1 isolate HTLV-III_{RF} in C3, MT-2 and H9 target cells is shown in Table 1. In the absence of Ampligen, four days after viral challenge in C3 and MT-2 cells and 6 days after viral challenge in H9 cells, all cells were expressing HIV-1 p24 antigen. This was associated with high levels of RT activity in culture fluids and dramatic reductions in viable cells. In contrast, with Ampligen present, few cells were positive for HIV-1 p24 expression, low or undetectable levels of RT activity were present, and there were no signs of cytopathic effect. Although the percent of p24 positive C3 and MT-2 cells given in Table 1 at 4 days is representative of less than 15% of the original cell population because of cytolysis, all cells were positive two days earlier at which time cytolysis was minimal. Using vital dye uptake and hemacytometer cell counts, we have found no toxicity of Ampligen at 50 μg/ml in any of these cell lines. Furthermore, in microtiter assays as described (Montefiori et al., 1987) Ampligen toxicity was not observed until exogenous concentrations greater than 50 μg/ml were employed (data not shown).

The antiviral activity of Ampligen against the HIV-1 isolates HTLV-III_B and HTLV-III_{RF} in U937 cells is shown in Fig. 1. Infection of U937 cells with HIV-1 proceeds much slower than in C3, MT-2 and H9 cells; therefore, samples for IIF and RT activity were obtained after 6, 8, 10 and 12 days of incubation. Also, HIV-

TABLE 1

Anti HTLV-III_{RF} activity of ampligen.

Cell line*	Ampligen (50 μg/ml)	Immuno-fluorescence (% positive)	Percent viable cells**	RT activity (cpm × 10^{-3}/ml)
C3	–	100[L]	14.5	2,369
	+	0.5	100	0
MT-2	–	100[L]	8.6	417
	+	2	100	10
H9	–	100	58	1,823
	+	0.5	100	8

*Cells were preincubated in the presence and absence of Ampligen for 18 h prior to viral challenge. Cells preincubated with Ampligen were continued to be incubated with Ampligen in the growth medium following viral challenge. The data is representative of at least two independent experiments with each cell line.

**Percent viable cells was determined by vital dye (neutral red) uptake as described (Montefiori and Mitchell, 1978b) relative to untreated, uninfected control cultures.

[L]Denotes extensive cytolysis.

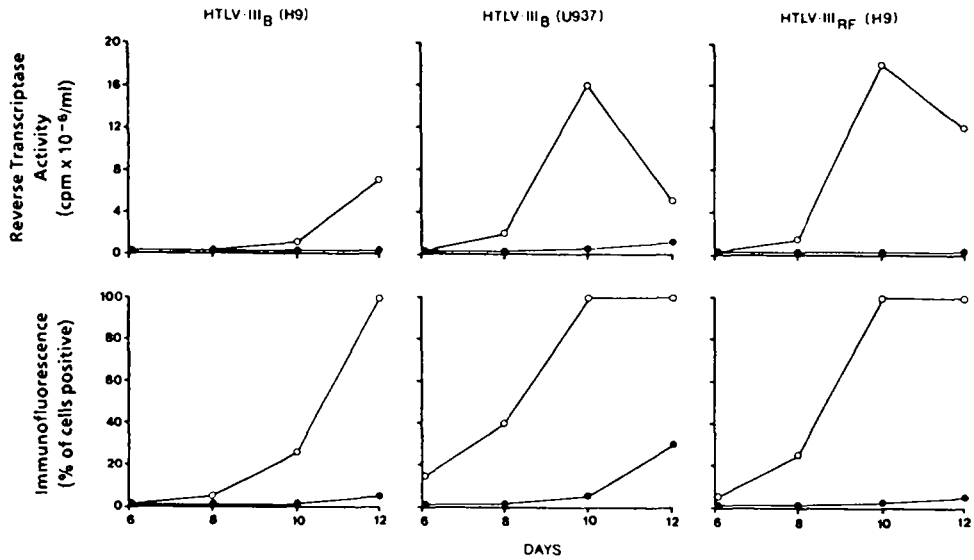

Fig. 1. Antiviral activity of Ampligen against the HIV-1 isolates HTLV-III_B and HTLV-III_{RF} in the monocyte/macrophage cell line U937. Duplicate cultures of U937 cells were preincubated in the presence and absence of Ampligen (50 μg/ml) for 18 hours and then challenged with either HTLV-III_B or HTLV-III_{RF} as designated. The cell line used as a source of virus is shown in parenthesis. Equal portions of cultures were harvested for IIF and RT at the days of incubation indicated. •, with Ampligen; ○, without Ampligen. Replicate cultures had values which always agreed within 15%.

1-induced cytopathic effect is mild in U937 cells; therefore, vital dye uptake was not used as a determinant for infection (i.e. fewer virus-producing cells at the time of analysis). In the absence of Ampligen, all cells were IIF positive for HIV-1 p24 expression after 12 days for HTLV-III_B produced in H9 cells and after 10 days for both HTLV-III_B produced in U937 cells and HTLV-III_{RF} produced in H9 cells. Also, in the absence of Ampligen, HIV-1 p24 expression was paralleled by a dramatic rise in RT activity. The decrease in RT activity which occurred after day 10 in U937 cultures infected with virus from U937/HTLV-III_B or H9/HTLV-III_{RF} cultures was due to a mild cytopathic effect observed early in infection (i.e., fewer virus-producing cells at the time of analysis). In striking contrast, the presence of Ampligen provided significant protection from infection. This was evident after 12 days of incubation when fewer than 5% of cells challenged with virus from H9/HTLV-III_B or H9/HTLV-III_{RF} cultures and fewer than 20% of cells challenged with virus from U937/HTLV-III_B cultures were positive for HIV-1 p24 antigen expression; furthermore, RT activities were greatly reduced or undetectable in all cultures infected in the presence of Ampligen.

An analysis was made to determine the minimum length of time required for cells to be preincubated with Ampligen in order to achieve maximum antiviral activity. The results of this analysis, using MT-2 cells as targets and H9/HTLV-III_B as a source of virus, are illustrated in Fig. 2. One set of cultures was preincubated

with Ampligen, then had Ampligen removed immediately following viral challenge so that antiviral activity would be a function of preincubation alone. A second set of cultures was preincubated with Ampligen, then continued to be incubated with Ampligen present following viral challenge. In both cases, antiviral activity was observed with as little as 5 min of preincubation for which 21% protection was provided by preincubation alone and 49% protection was provided by preincubation and continued incubation with Ampligen. Also, in both cases maximum antiviral activity was observed with a 1 h preincubation period.

Discussion

The experiments described in this report address whether either HIV-1 genomic variation or host cell range will affect Ampligen's activity against this virus. These were important issues to address since HIV-1 demonstrates a high degree of genomic variation (Hahn et al., 1985) and infects cells of monocyte/macrophage lineage in addition to T-helper lymphocytes (Klatzmann et al., 1984; Koenig et al.,

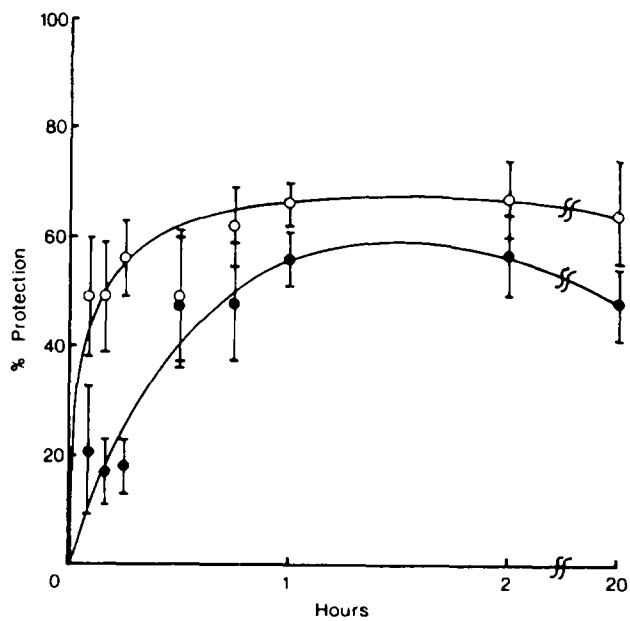

Fig. 2. Effect of various preincubation times on Ampligen's anti-HIV-1 activity. Cultures of MT-2 cells (5×10^5 cells/ml) in 25 cm^2 culture flasks were preincubated with and without Ampligen (50 μg/ml) for the various times indicated. Cells from one-half of each culture were then washed with RPMI-1640 to remove Ampligen and suspended in an equal volume of Ampligen-free growth medium. The cells from each set of cultures were then challenged with HTLV-III_B produced in H9 cells at an m.o.i. of 1–5 in 96-well microtiter plates. Plates were assayed for cytopathic effect after 4 days of incubation. Cells incubated in the absence of Ampligen were used for the uninfected and infected controls in these experiments. ○, ampligen present continuously; ●, Ampligen removed after the preincubation periods.

1986; Wong-Staal and Gallo, 1985). Such findings suggest that effective therapeutic intervention in HIV-1 infection will require broad spectrum applicability.

HIV-1 genomic variation did not appear to be an obstacle since Ampligen was active against the diverse isolates HTLV-III_B (which is actually a mixture of at least two similar variants (Shaw et al., 1984) and HTLV-III_{RF} (Table 1 and Fig. 1). Ampligen was shown previously to be active against the French isolate, LAV (Montefiori and Mitchell, 1987b). In addition, Ampligen was effective against HTLV-III_B produced in two different cell lines, H9 and U937 (Fig. 1), while it was also effective against HTLV-III_B produced in CEM and C3 cells and against LAV produced in H9, CEM and C3 cells (data not shown). These latter results demonstrate a lack of influence that variations in cell type might have had (i.e., cell-specified modifications such as protein glycosylation) on Ampligen's anti-HIV-1 activity.

We also examined Ampligen's effectiveness in three different helper T-cell lines (C3, MT-2 and H9) and in a monocyte/macrophage cell line (U937). In addition to Ampligen's activity against HTLV-III_B in C3 cells and against LAV in C3 and CEM cells reported earlier (Montefiori and Mitchell, 1987a), we found it to be effective against HTLV-III_{RF} in MT-2, H9 and U937 cells and against HTLV-III_B in U937 cells (Table 1 and Fig. 1). Thus, as far as Ampligen's anti-HIV-1 activity is concerned, cell type appears to have no effect either on the susceptibility of the infecting virions or on the HIV-1 target. In light of recent evidence that cells of monocyte/macrophage lineage may be responsible for AIDS-associated encephalopathy (Koenig et al., 1986), it was encouraging to find that U937 cells were responsive to the anti-HIV-1 activity of Ampligen. Likewise, virus produced in U937 cells was equally susceptible to Ampligen's activity. U937 cells appear to exist in a relatively inactive state, a finding supported by the long period required for HIV-1 expression (10 days) and the ability to be activated in vitro with mitogens (Larrick et al., 1980).

A final aspect of this study was the effect of various preincubation times on Ampligen's anti-HIV-1 activity. Previously, we have allowed 18–24 h of preincubation in anticipation of a need for IFN to be synthesized, secreted and accumulated in the medium before activity could be detected (Montefiori and Mitchell, 1987b). However, full activity was observed in MT-2 cells with a 1-h preincubation period, while partial activity was observed with as little as 5 min preincubation prior to virus challenge (Fig. 2). Perhaps Ampligen's activation of uninduced, constitutive levels of IFN-inducible, dsRNA-dependent enzymes accounts for this early activity. A more likely explanation, however, is that the preincubation periods given in this report should also include the time required for HIV-1 adsorption, penetration, uncoating and replication since IFN acts late in the replicative cycle (Lengyel, 1982) In MT-4 cells, a cell line closely related to MT-2 (Harada et al., 1985a), this time frame is on the order of 12–24 h (Harada et al., 1985b), thus allowing sufficient time for IFN-mediated antiviral activity to occur in response to dsRNA addition. Our data do not rule out the possibility of Ampligen remaining adhered to cell membranes or stored as intracellular pools after washing.

In conclusion, we have presented evidence that Ampligen's anti-HIV-1 activity in vitro is not limited by the highly mutable nature or host cell range of HIV-1.

This was indicated by Ampligen's activity against the divergent HIV-1 isolates HTLV-III_B and HTLV-III_{RF}, as well as its ability to establish an anti-HIV state in three different helper T-cell lines (C3, MT-2 and H9) and in a monocyte/macrophage cell line (U937). Furthermore, as little as 1 h preincubation with Ampligen was required for full anti-HIV-1 activity in vitro. The spectrum of effectiveness against HIV-1 observed for Ampligen in these in vitro studies parallels the apparent effectiveness of Ampligen in the clinical data reported to date. Thus, Ampligen appears to offer significant potential clinical effectiveness in the treatment of HIV-1 infection.

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References

Carter, W.A. and DeClercq, E. (1974) Viral infection and host defense. Science 186, 1172–1178.

Carter, W.A., Brodsky, I., Pellegrino, M.G., Henriques, H.F., Parenti, D.M., Schulof, R.S., Robinson, W.E., Volsky, D.J., Paxton, H., Kariko, K., Suhadolnik, R.J., Strayer, D.R., Lewin, M., Einck, L., Simon, G.L., Scheib, R.G., Montefiori, D.C., Mitchell, W.M., Paul, D., Meyer, W.A., III, Reichenbach, N. and Gillespie, D.H (1987) Clinical, immunological, and virological effects of Ampligen, a mismatched double-stranded RNA, in patients with AIDS or AIDS-related complex. Lancet i, 1286–1292.

Haertle, T., Carrera, C.J., McDougal, J.S., Sowers, L.C., Richman, D.D. and Carson, D.A. (1988) Metabolism and anti-HIV activity of 2-halo 2',3'-dideoxyadenosine derivatives. J. Biol. Chem. In Press.

Hahn, B.H., Gonda, M.A., Shaw, G.M., Popovic, M., Hoxie, J.A., Gallo, R.C. and Wong-Staal, F. (1985) Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III: different viruses exhibit greatest divergence in their envelope genes. Proc. Natl. Acad. Sci. USA 82, 4813–4817.

Harada, S., Koyanagi, Y. and Yamamoto, N. (1985a) Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 229, 563–566.

Harada, S., Koyanagi, Y. and Yamamoto, N. (1985b) Infection of the human T-lymphotropic virus type-I (HTLV-I)-bearing MT-4 cells with HTLV-III (AIDS virus): Chronological studies of early events. Virology 146, 272–281.

Hearl, W.G. and Johnston, M.I. (1986) A misaligned double-stranded RNA, poly(I)·poly (C_{12},U), induces accumulation of 2',5'-oligoadenylates in mouse tissues. Biochem. Biophys. Res. Commun. 138, 40–46.

Hubbell, H.R., Kvalnes-Krick, K., Carter, W.A. and Strayer, D.R. (1985) Antiproliferative and immunomodulatory actions of interferon-β and double-stranded RNA, individually and in combination, on human bladder tumor xenographs in nude mice. Cancer Res. 45, 2481–2486.

Klatzmann, D., Barre-Sinoussi, F., Nugeyre, M.T. Gauguet, C., Vilmer, E. Griscelli, C., Brun-Vezinet, F., Rouzioux, C., Gluckman, J.C., Chermann, J.-C. and Montagnier, L. (1984) Selective tropism of lymphadenopathy associated virus (LAV) for helper inducer T-lymphocytes. Science 225, 59–63.

Koenig, S., Gendelman, H.E., Orenstein, J.M., Dal Canto, M.C., Pezeshkpour, G.H., Yungbluth, M., Janotta, F., Aksamit, A., Martin, M.A. and Fauci, A.S. (1986) Detection of AIDS virus in

macrophages in brain tissue from AIDS patients with encephalopathy. Science 233, 1089–1093.
Larrick, J.W., Fischer, D.G., Anderson, S.J. and Koren, H.S. (1980) Characteristics of a human macrophage-like cell line stimulated in vitro: a model of macrophage functions. J. Immunol. 125, 6–12.
Lengyel, P. (1982) Biochemistry of interferons and their actions. Ann. Rev. Biochem. 51, 251–282.
Mitchell, W.M., Montefiori, D.C., Robinson, W.E., Jr., Strayer, D.R. and Carter, W.A. (1987) Mismatched double-stranded RNA (Ampligen) reduces concentration of Zidovudine (azodithymidine) required for in vitro inhibition of human immunodeficiency virus. Lancet i, 890–892.
Montefiori, D.C. and Mitchell, W.M. (1986) Infection of the HTLV-II bearing T cell line C3 with HTLV-III/LAV is highly permissive and lytic. Virology 155, 726–731.
Montefiori, D.C. and Mitchell, W.M. (1987a) Persistent coinfection of T lymphocytes with HTLV-II and HIV and the role of syncytium formation in HIV induced cytopathic effect. Virology 160, 372–378.
Montefiori, D.C. and Mitchell, W.M. (1987b) Antiviral activity of mismatched double-stranded RNA against human immunodeficiency virus in vitro. Proc. Natl. Acad. Sci. USA 84, 2985–2989.
Montefiori, D.C., Robinson, W.E., Jr., Schuffman, S.S. and Mitchell, W.M. (1988) Evaluation of antiviral drugs and neutralizing antibodies against human immunodeficiency virus by a rapid and sensitive microtiter infection assay. J. Clin. Microbiol. 26, 231–235.
Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, J.D. and Gallo, R.C. (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA 77, 7415–7419.
Popovic, M., Kalyanaraman, V.S., Mann, D.L., Richardson, E., Sarin, P.S. and Gallo, R.C. (1984a) Infection and transformation of T cells by human T-cell leukemia/lymphoma virus of subgroups I and II (HTLV-I, HTLV-II). In: Human T-Cell Leukemia/Lymphoma Virus (Gallo, R.C., Essex, M.E. and Gross, L. eds.), Cold Spring Harbor, pp. 217–227.
Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984b) Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497–500.
Shaw, G.M., Hahn, B.H., Arya, S.K., Groopman, J.E., Gallo, R.C. and Wong-Staal, F. (1984) Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science 226, 1165–1171.
Sundstrom, C. and Nilsson, K. (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U937). Int. J. Cancer 17, 565–577.
Wong-Staal, F. and Gallo, R.C. (1985) Human T-lymphotropic retroviruses. Nature (London) 317, 395–403.