ANTIVIRAL CHEMOTHERAPY[1,2]

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INTRODUCTION

Most of the acute infectious diseases of man are caused by viruses. Chemotherapy, which has been spectacularly successful against bacterial infections, has until very recently been unavailable for virus diseases; in fact, for a long time the theoretical feasibility of antiviral chemotherapy was seriously questioned. The reason for this doubt was that the reproduction of a virus was thought to be totally dependent on the metabolic machinery of the host cell. The cell was thought to provide not only the "building blocks" for the virus—low-molecular precursors for synthesis of viral nucleic acid and protein—but also all the biosynthetic and energy-supplying mechanisms required by the virus for its replication. It was therefore thought that there was no way of inhibiting viral biosynthesis selectively, i.e., without at the same time inhibiting some aspect of cellular biosynthesis. In the laboratory, it proved very easy to inhibit virus multiplication. This could be done by interfering with the energy-yielding reactions of the cell, or by introducing analogues of purines, pyrimidines, or amino acids. However, such measures inhibited not only viral biosynthesis, but cellular biosynthesis as well. These facts provided support for the belief that successful antiviral chemotherapy could not be expected.

Recent progress in basic animal virology has shown that these doubts are not fully justified. Virus-directed enzymes have been discovered which function in the synthesis of viral nucleic acid. In cell culture systems, selective inhibition of virus multiplication has been clearly demonstrated, and successful clinical antiviral chemotherapy and chemoprophylaxis have been recorded. The main purpose of this article is to describe some of these new developments, which indicate that there do exist targets for antiviral chemotherapy which are virus-specific. A complete survey of the field of antiviral chemotherapy will not be attempted; the interested reader may consult some of the more comprehensive reviews (1–6a).

VIRAL REPRODUCTION

To understand the theoretical basis of antiviral chemotherapy, it is necessary to be familiar with the major features of viral reproduction (7). Vi-

[1] The survey of the literature pertaining to this review was concluded in June 1965.

[2] The following abbreviations will be used: IUdR (5-iodo-2′-deoxyuridine); FUdR (5-fluoro-2′-deoxyuridine); BUdR (5-bromo-2′-deoxyuridine); HBB [2-(α-hydroxybenzyl)-benzimidazole].

ruses consist of nucleic acid—either DNA or RNA—which is surrounded by a protective protein shell. The nucleic acid is the genetic material of the virus, the viral genome. The DNA of animal viruses is double-stranded, while the RNA of animal viruses may be either single- or double-stranded. The viral protein coat has reactive sites capable of combining with cellular receptors and thus allowing the virus to attach to a susceptible host cell. The initial steps of the virus-cell interaction are not well understood, but, after the infecting virus has been taken up by the cell, the viral genome is released from its shell, enters the cytoplasmic or nucleoplasmic matrix proper, and proceeds to direct the synthesis of various virus-specific macromolecules.

The viral genome of single-stranded RNA viruses has been shown to act itself as messenger RNA in the synthesis of virus-directed proteins, whereas double-stranded viral DNA or RNA cannot perform this function. When the viral DNA is double-stranded, a single-stranded messenger RNA is synthesized with the DNA as template. Presumably, a similar mechanism operates in viruses with double-stranded RNA. It is not yet known which enzymes function in the synthesis of messenger RNA on viral double-stranded DNA or RNA; it may be the cellular RNA polymerase.

The viral messenger RNA, using the protein-synthesizing apparatus of the cell, directs the synthesis of various proteins: (*a*) inhibitors of cellular macromolecular synthesis; (*b*) new enzymes which function in viral nucleic acid synthesis; (*c*) viral coat proteins; and (*d*) repressors which function in the regulation of viral biosynthesis.

With the appearance of a new virus-directed RNA polymerase in the infected cell, the viral genome of small RNA viruses is replicated. It remains to be seen whether single-stranded RNA, or a double-stranded "replicative" form which can be isolated from the infected cells, functions as template. The DNA of DNA viruses appears to replicate in a semiconservative manner. It is not yet known whether a new virus-directed DNA polymerase is synthesized in virus-infected mammalian cells. The mode of replication of RNA of double-stranded RNA viruses and its enzymatic mechanism remain to be elucidated.

In summary, in the virus-infected cell, various virus-directed macromolecules are synthesized. Finally, the viral particle matures, that is it is assembled from virus precursor molecules, proteins, nucleic acid, and sometimes lipids, to form a new, infectious particle. The viral progeny is released from the cell to a variable extent, depending on the virus-cell system, and a new infective cycle can begin.

VIRAL SPECIFICITY AND TARGETS FOR VIRUS-SELECTIVE INHIBITORS

The evidence cited above indicates that despite the dependence of viral biosynthesis on the host cell, the viral genome directs the synthesis of a variety of virus-specific macromolecules. It is postulated that the specific

configuration of these viral macromolecules may provide virus-specific targets for selective inhibitors of virus multiplication. Models of this kind are now well known to the enzyme chemist; small-molecular metabolites have been shown to regulate the activity of enzymes, probably by altering the three-dimensional structure of the enzyme and thus changing the fit of the substrate-enzyme complex (8). Similarly, it is conceivable that small molecular compounds may "recognize" particular sites in virus-specific macromolecules, attach to them, and thus selectively impede the proper functions of virus-specific macromolecules.

Interactions of inhibitory compounds with viral macromolecules may take place at various stages of the viral infective cycle. If chemical substances combine with the viral protein coat, either attachment of the virus to the host cell or virus penetration may be inhibited. In nature, such reactions are well known as exemplified by the interaction of the viral protein coat with specific antibodies or mucoprotein inhibitors. Antibodies react specifically with a critical site on the surface of a virus particle, whose configuration is specific for virus of a particular immunological type. Depending on the number of antibody molecules attached to the specific sites on the virus protein coat, virus attachment or penetration is inhibited.

It is possible, also, that chemical compounds can interact with viral nucleic acid. If this were the case, e.g., with the RNA of small RNA viruses, two functions might be inhibited: (*a*) the template function in the replication of the viral genome, (*b*) the messenger function in the synthesis of virus-directed proteins. Finally, selective inhibitors might interfere with the functions of virus-directed enzymes or their morphogenesis, i.e., the assembly of enzyme subunits, the monomers, to the functional oligomer.

If these assumptions are correct, it should be possible to find virus-selective inhibitors, and, as already indicated, several such substances have in fact been discovered in recent years. In the present review, there is discussed in some detail the status of the mechanisms of action of these virus-selective compounds.

There is one other possible approach to antiviral chemotherapy. It was mentioned above that numerous structural analogues of naturally occurring metabolites common to virus and host cell, examined in the past, did not provide a rational approach to selective inhibition of virus multiplication. It has recently been shown, however, that in the infected cell, new enzymes are synthesized under the control of the viral genome. There is, therefore, the possibility that such virus-directed enzymes may be more sensitive to certain structural analogues than the corresponding enzymes in the uninfected cell. This whole question has already come up in connection with the effects of halogenated deoxyuridines on the multiplication of DNA viruses. In the first part of the discussion which follows, we shall therefore consider in some detail the action of certain structural analogues of nucleosides and amino acids on viral multiplication. In the later sections, the virus-specific effects of a number of selective inhibitors of virus multiplication will be reviewed and analyzed.

STRUCTURAL ANALOGUES OF CONSTITUENTS OF DNA AND PROTEINS

5-Iodo-2′-Deoxyuridine and 5-Bromo-2′-Deoxyuridine

It has been known for many years that structural analogues of constituents of DNA, e.g., the halogenated pyrimidine nucleosides, would not only affect DNA synthesis both in bacterial and animal cells, but would also inhibit the multiplication of DNA-containing animal viruses. Thus, iodoxuridine (5-iodo-2′-deoxyuridine; IUdR) inhibits the multiplication of DNA viruses including SV40 virus (9), adenoviruses (10), herpes virus (11), varicella-zoster virus (12), and vaccinia virus (11). RNA-containing viruses, with the possible exception of Rous sarcoma virus (13), are resistant to the action of IUdR (11). Because of their effects on DNA metabolism, no appreciable virus-selective action of these compounds was expected and, in fact, 5-fluoro-2′-deoxyuridine (FUdR) failed to reveal significant selectivity with respect to the reproduction of herpes virus in a HeLa cell culture system (14). Nevertheless, Kaufman subsequently reported the successful treatment of herpes simplex virus (15, 16) and vaccinia virus keratitis (17) with IUdR in rabbits, and of herpes keratitis in man. 5-Bromo-2′-deoxyuridine (BUdR) also proved effective in the treatment of experimental herpetic keratitis, whereas FUdR was found ineffective (15).

Herpetic keratitis is a serious eye disease, frequently leading to loss of vision. Following Kaufman's report, therefore, IUdR treatment of this condition was tried by a great number of ophthalmologists. In general, the various reports, which include reports of double-blind studies, agree that IUdR is effective in the treatment of dendritic keratitis [for reviews see (18, 19)]. IUdR treatment may also be indicated to minimize complications of corticosteroid therapy of metaherpetic or disciform keratitis, such as recurrences of superficial corneal lesions and perforation of the cornea (20, 21). The effect of IUdR on stromal disease remains to be evaluated (18, 19, 22, 23). Clinically, the questions as to how IUdR therapy of herpes of the eye compares with the older forms of treatment seem not yet satisfactorily answered.

These findings raise two important questions: (*a*) What is the mechanism of action of IUdR, in particular, its virus-inhibitory action? and (*b*) what is the basis of the apparent virus-selectivity of IUdR? The same questions may be asked about BUdR.

Mode of action of IUdR and BUdR.—IUdR, like BUdR, is first phosphorylated in the cell to IUdR-5′-monophosphate by the enzyme thymidine kinase. It may then be converted by appropriate kinases into the di- and triphosphate form. Finally, both of these drugs may be incorporated as 5′-triphosphates into DNA in place of thymidine. This has been demonstrated in mammalian cells (24-28) and in various bacteria (29, 30, 31). The drugs are likewise incorporated into the DNA of bacterial viruses (30,

32) and animal viruses (33, 34, 35). There is a striking structural similarity between these halogenated analogues and thymidine, and therefore the incorporation into DNA of IUdR or BUdR, instead of thymidine, can occur readily. However, the van der Waals radii of the 5-substituents in IUdR, BUdR, and thymidine, viz., I, Br, and CH_3, are not identical, and the distribution of electrons in IUdR and BUdR is disturbed because of the substitution of the halogen (36, 37). It is not surprising that incorporation of the halogenated precursors into DNA causes a number of chemical and biological effects.

Incorporation of IUdR or BUdR into DNA results in a significant increase in the temperature of helix-coil transition. This finding has been related to physicochemical changes in the structure of DNA which might also impair the separation of the complementary DNA strands during DNA replication, with resulting inhibition of DNA synthesis (36, 37). Replacement of thymidine by IUdR or BUdR in DNA also leads to a significant increase in the rate of mutation, especially in some bacteria and phages. This has been attributed to an increase in the probability of base pairing errors during DNA replication (38, 39). Similarly, increased errors in protein formation would be expected because of mistakes in the formation of messenger RNA.

Besides the effects of IUdR or BUdR which result from their incorporation into DNA, these compounds per se, or after phosphorylation, may inhibit various enzymatic reactions in DNA synthesis. Delamore & Prusoff (40) studied the effects of IUdR on the biosynthesis of phosphorylated derivatives of thymidine in various murine and neoplastic tissues. One important result of this investigation was the demonstration that IUdR and its phosphorylated derivatives exert inhibitory effects at various reaction sites and that the metabolic sites primarily affected vary with the different tissues studied. Prusoff, Bakhle & Sekely (41) suggest that IUdR-5′-triphosphate may also inhibit those enzymes over which deoxythymidine-5′-triphosphate normally exerts a regulatory role. BUdR-5′-triphosphate was shown to inhibit thymidine kinase of *Escherichia coli* in a manner similar to deoxythymidine-5′-triphosphate (42).

In view of this wide variety of possible effects of IUdR and BUdR, it is not surprising that the overall effects of these compounds on various organisms differ somewhat from one organism to another (25, 26, 28, 32, 43, 44).

Effects of IUdR and BUdR on animal virus reproduction.—We may now consider what is known of the mechanism of action of IUdR and BUdR on the replication of animal viruses, in particular that of herpes virus. As expected, IUdR has no direct effect on extracellular herpes virus (23) nor does it interfere with adsorption of the virus to cells (45). In IUdR-treated, herpes virus-infected cells there is no detectable production of infectious virus, but large amounts of viral components, presumably viral DNA and proteins, are synthesized, as revealed by acridine orange and fluorescent antibody staining. The intensity of staining is about equal

in untreated and IUdR-treated infected cultures. In addition, ragged, naked, structurally imperfect particles are seen by electron microscopy in the treated cultures. They amount to about 22 per cent of the particles observed in untreated cultures. Since no infectious virus is produced, the unusually high virus particle: infectious virus ratio of 10^7, results (46).

Kaplan, Ben-Porat & Kamiya (47) have studied the effects of IUdR and BUdR on the replication of pseudorabies virus, an agent belonging to the herpes virus group. The synthesis of pseudorabies virus DNA occurs at about the same rate in drug-treated cells as in control cells. Both IUdR and BUdR are incorporated into viral DNA. Viral antigen also accumulates in the drug-treated cells. Despite the synthesis of viral macromolecules, practically no infectious virus is made. Assembly of noninfectious viral particles occurs in BUdR-treated cells, but not in IUdR-treated cells. It has been shown in elegant experiments (47) that this lack of assembly of viral particles is not caused by the presence in the viral DNA of IUdR per se, but probably by the formation of faulty viral proteins. Lastly, Kaplan, Ben-Porat & Kamiya (47) demonstrated that substitution of IUdR or BUdR for thymidine in viral progeny DNA interferes with the mechanisms regulating the level of activity of enzymes involved in DNA synthesis.

In herpes virus-infected, BUdR-treated HeLa cells, intranuclear DNA-containing inclusions form and viral antigen accumulates in a pattern indistinguishable from that in untreated cells (48). Furthermore, although in adenovirus-infected BUdR-treated cells, the production of infectious virus is markedly inhibited, the synthesis of the three soluble adenovirus antigens does take place, and noninfectious particles with the morphological characteristics of adenovirus particles are formed (49, 50). Likewise, in the presence of BUdR, vaccinia virus-infected cells yield large quantities of noninfectious, malformed particles (33). The DNA isolated from virus grown in the presence of BUdR is smaller in molecular weight and is heterogeneous in density compared to the DNA of control virus. It is not known whether the fragmentation of the DNA molecules occurs before or after DNA extraction.

In summary, IUdR and BUdR appear to permit the accumulation of large quantities of viral precursor molecules in DNA virus-infected cells. These may or may not be partially assembled to yield malformed particles. Also, the regulation of enzymatic activity seems to be disturbed in the infected cell. The most plausible hypothesis to explain these findings would be that incorporation of these drugs into the DNA polymer leads to miscoding of messenger RNA with synthesis of faulty proteins. Incorporation of IUdR and BUdR into the DNA of vaccinia and pseudorabies virus has already been demonstrated (33, 34, 35, 47), which supports this hypothesis. Incorporation of IUdR into herpes virus DNA has not yet been detected, although the low sensitivity of the method used would not exclude relatively small but significant incorporation (51). Some decrease in viral DNA biosynthesis seems likely in IUdR- or BUdR-treated cells, at least in

some instances; nevertheless, as reviewed above, substantial amounts of viral DNA appear to be made. Furthermore, as discussed below, biochemical studies do not reveal any irreversible inhibition of thymidine or thymidylic acid kinase from herpes virus-infected cells by IUdR or IUdR-5′-monophosphate (41).

Virus selectivity of IUdR and BUdR.—Prusoff, Bakhle & Sekely (41) have attempted to find a possible biochemical mechanism for the postulated virus-selective action of IUdR or BUdR. There is some evidence that following infection by vaccinia or herpes virus, virus-specific enzymes may be synthesized (52, 53, 54). Therefore, Prusoff, Bakhle & Sekely (41) compared the thymidine and thymidylic acid kinase activities in herpes virus-infected and in control cells. Under various treatments, these enzyme activities in extracts from herpes virus-infected cells revealed no differences from those of uninfected cells. The sensitivities of these enzyme activities to inhibition by IUdR or IUdR-5′-monophosphate, respectively, were identical for infected and uninfected cells. The effect of IUdR-5′-triphosphate on DNA polymerase remains to be investigated.

Thus, biochemical studies do not yet reveal any virus selectivity of IUdR. This is in accordance with investigations in which the sensitivities of DNA virus multiplication and cell division to IUdR or BUdR were compared (12, 23, 48, 49, 55). All of these studies demonstrate that IUdR and BUdR have low selectivity or no selectivity at all for various DNA viruses.

It has been suggested, therefore, (56) that the chemotherapeutic effects of IUdR or BUdR in the eye may be caused by kinetic and quantitative differences between viral and cellular DNA synthesis. Viral DNA synthesis is continuous and rapid, while the DNA synthesis of the uninfected cell is intermittent. Therefore, virus reproduction would be severely affected by the drug, but most of the uninfected cells would not, particularly in the absence of a high rate of cell division. Certainly, with prolonged treatment, some cells would incorporate considerable amounts of the analogues, which may ultimately prove lethal to these cells. It is not surprising, therefore, that IUdR seems to affect epithelial regeneration and wound healing of larger defects in the cornea (57, 58, 59). Uncontrolled use of these analogues may cause severe complications. It is not known whether the well-established mutagenic activity of IUdR is of medical significance in connection with topical treatment in the eye.

The low virus selectivity of IUdR makes it inadvisable to administer this drug systemically, although it has been shown to interfere with the development of dermal lesions caused by vaccinia virus in the rabbit (60). Calabresi, McCollum & Welch (60) have suggested that chemotherapeutic trials with IUdR may be indicated only in potentially lethal virus diseases such as herpes virus encephalitis or simian B virus infection.

Effects of IUdR and BUdR on virus-induced cell damage.—Although IUdR treatment of cell cultures infected with DNA viruses can prevent virus spread to uninfected cells because of inhibition of virus synthesis (12,

61), IUdR and BUdR do not save the individual infected cell from ultimate death (45, 48, 49). This result is not unexpected, since virus-specific macromolecules and even incomplete virus particles accumulate in the infected cell despite the treatment. These virus-controlled syntheses lead to derangements of cell metabolism, which are lethal.

Viral mutants resistant to or dependent on IUdR and BUdR.—IUdR-resistant virus occurs after IUdR treatment of patients with herpetic keratitis (18, 62) and can readily be obtained in the laboratory by passing herpes or vaccinia virus in the presence of the inhibitor (63–70). The resistant virus appears to be genetically stable. The mechanism of IUdR resistance is not known. It is of clinical importance that IUdR-resistant herpes virus mutants are still sensitive to other analogues of DNA metabolism such as cytosine arabinoside (64, 65, 71) and 5-trifluoromethyl-2′-deoxyuridine (72).

The development of IUdR-dependent herpes virus apparently has not been observed. However, Stevens & Groman (73) have reported that infectious bovine rhinotracheitis virus grown once in the presence of BUdR plus FUdR, yields virus with a plaque count about 40 to 90 times higher in the presence of these compounds than in their absence.

Cytosine Arabinoside, 5-Trifluoromethyl-2′-deoxyuridine, and 5-Methylamino-2′-deoxyuridine

Besides agents such as 5-iodo-2′-deoxycytidine (74), which are metabolized to IUdR and serve as a reservoir of this less thermostable and less soluble compound, other antimetabolites interfering with DNA synthesis have been tried in the treatment of herpetic keratitis. Cytosine arabinoside (1-β-D-arabinofuranosylcytosine hydrochloride; cytarabine) inhibits the multiplication of many DNA viruses (64, 75) and appears to be effective in the treatment of herpetic keratitis in rabbits (71, 76, 77) and man (78). However, cytosine arabinoside appears to be more toxic for the eye than IUdR (79). IUdR and cytosine arabinoside have an additive action in the treatment of experimental herpetic keratitis (80). Cytosine arabinoside appears not to be incorporated into DNA, but probably affects various sites in the metabolic pathway from cytidylic acid to DNA (75, 81, 82).

5-Trifluoromethyl-2′-deoxyuridine also has potent therapeutic antiviral activity in experimental herpetic keratitis (72). Like cytosine arabinoside, it also is capable of inhibiting IUdR-resistant herpes virus. The mechanism of the antiviral action of 5-trifluoromethyl-2′-deoxyuridine remains to be elucidated, but the compound has been shown to be incorporated into the DNA of bacteriophage T4 and to be mutagenic (83).

5-Methylamino-2′-deoxyuridine has recently been shown to be therapeutically active in experimental herpetic keratitis of rabbits at nontoxic concentrations (83a). The compound appears to be incorporated into the DNA of host cells to a lesser extent than IUdR.

p-Fluorophenylalanine

A limited chemotherapeutic trial in herpetic keratitis has also been made with another metabolite, the amino acid analogue *p*-fluorophenylalanine (84). The effectiveness of this treatment remains to be determined. *p*-Fluorophenylalanine appears to be incorporated into proteins (85), thus causing the synthesis of fraudulent, functionally impaired proteins (86). Depending on where the analogue is incorporated into the protein in relation to active sites, a differential sensitivity of various proteins to the analogue is to be expected and has in fact been demonstrated for various poliovirus-directed proteins (86, 87, 88). More quantitative studies on virus selectivity need to be done.

Despite the theoretical possibility of preferentially affecting virus-specific enzymes with metabolic analogues and thus achieving virus selectivity, it is apparent from the preceding discussion that this has not yet been accomplished in the laboratory. In the following sections, we shall discuss inhibitors of virus reproduction which exhibit remarkable selectivity in cell culture systems and sometimes also in whole organisms.

SELECTIVE INHIBITORS OF VIRUS MULTIPLICATION

Amantadine

Amantadine (1-adamantanamine) is a symmetrical heterocyclic amine. Its virus-inhibitory action was discovered in an industrial screening program. It selectively inhibits the multiplication of certain myxoviruses in cell culture and *in ovo*, and has shown a protective effect in influenza virus infections in mice and in man (89, 90, 91). Myxoviruses are lipid-containing RNA viruses of medium size. They consist of a central nucleoprotein helix surrounded by an envelope which is covered with projections. They have been divided into two subgroups on the basis of biological and morphologic properties: subgroup 1, which includes influenza and fowl plague viruses, and subgroup 2, which includes mumps, Newcastle disease, and parainfluenza viruses.

The following viruses have been found susceptible to amantadine in cell culture: one or more representatives of influenza A, A_1, A_2, and C viruses, parainfluenza 1 (Sendai) virus, and rubella virus (92, 93). (Rubella virus has not yet been assigned to any of the major virus groups, but possesses some of the characteristics of myxoviruses.) Amantadine has also been reported to inhibit pseudorabies virus, a member of the herpes virus group, in cell culture (92), but it did not protect mice after intravenous inoculation of this virus (94). The following viruses were found amantadine-insensitive in cell culture or *in ovo:* several picornaviruses, arboviruses, reovirus, Rous sarcoma virus, parainfluenza virus 1 (C35), Newcastle disease virus, mumps and measles viruses, myxoma virus, two adenovirus types, herpes simplex virus, vesicular stomatitis virus, and vaccinia virus (89, 92, 93).

The reports concerning influenza B, parainfluenza 2 and 3, and respiratory syncytial viruses are apparently conflicting (92, 95), but these differences may be caused by differences in experimental procedure and in criteria of drug sensitivity and insensitivity.

Amantadine is nontoxic to cells at virus-inhibitory concentrations, when toxicity is determined by the morphological appearance and rate of multiplication of cells over a three-day period (92).

There is some evidence that amantadine interferes with an early step in virus-cell interaction, perhaps with virus penetration (89). It is reported that the compound causes no direct inactivation of viruses and that it does not significantly inhibit virus adsorption or the enzymatic release of virus from red blood cells (89, 96).

The antiviral effects of amantadine in mice correlate well with the results of cell culture studies. The administration of the compound to mice infected with small doses of influenza A, A_2, or Sendai virus increased the percentage of survivors and prolonged the survival time (89, 94). In general, to be effective, the compound had to be given shortly before virus inoculation; but a single dose, either intraperitoneal, subcutaneous, or oral, sufficed.

Bleidner et al. (97) studied the drug dynamics of amantadine in the mouse. Following a single oral dose ranging from 1 mg/kg to 100 mg/kg, 63 per cent of the compound was excreted unchanged in the urine. The rate of excretion appeared to be first-order, and the half-life time was about two hours. Excretion was virtually complete in 12 hours. A relatively high concentration of the drug was found in the lungs compared with blood and heart muscle.

Of great interest is the finding that in ferrets, amantadine aggravated rather than alleviated infection with influenza virus (95). The reasons for this finding are not clear.

In man, the antiviral effects of amantadine have been studied by determining the serological response in volunteers after administration of influenza A_2 virus (90, 91). Under these experimental conditions, the antibody response is a valuable parameter of the infectious process, since it is dependent on infection and viral multiplication. Pretreatment with amantadine, beginning 18 hours before virus inoculation, reduced by 30 to 50 per cent the serological response to infection among susceptible subjects. There was also a decrease in clinical influenzal illness in the volunteers. If clinical manifestations did appear, no alteration in the severity of the disease was observed. If treatment was begun four hours after virus inoculation, no antiviral effect was found.

In a prophylactic trial of amantadine in naturally occurring human influenza, the compound reduced the occurrence of clinical disease and of serological response to the virus as compared with a placebo (96, 98). The compound does not seem to interfere with antibody production per se.

In summary, the results obtained with amantadine in mice and the clini-

cal experiences are in good agreement with the hypothesis that the compound affects an early step in virus-cell interaction.

In man, amantadine is well absorbed by the oral route. There is no evidence of metabolism of the drug in man. As in the mouse, the major route of excretion appears to be in the urine, with an average recovery of 86 per cent. The half-life time in man is about 20 hours, which is considerably longer than in the mouse (97).

Grunert, McGahen & Davies (94) did not obtain drug-resistant influenza A (Swine/S 15) virus upon serial passage of the virus in treated mice. On the other hand, by growing influenza A_2 virus (Japan 305) in primary calf kidney cell cultures in the presence of amantadine, Cochran et al. (95) readily obtained drug-resistant virus. It is of interest that this amantadine-resistant virus was found also resistant to α-amino-*p*-methoxyphenyl-methanesulfonic acid, a compound which appears to inhibit an early step in virus replication (99, 100).

2-(α-Hydroxybenzyl)-Benzimidazole and Guanidine

The discovery of the selective virus-inhibitory activity of 2-(α-hydroxybenzyl)-benzimidazole (HBB) was the result of a systematic study on the relationship between the structure of a large number of benzimidazole derivatives and their virus-inhibitory activity (3, 101–104). The virus-inhibitory effect of guanidine was discovered in various programs in the search for antiviral agents (105–107).

HBB (108–112) and guanidine (105, 110–115) inhibit the multiplication of many members of the picornavirus group. These are small, lipid-free RNA viruses of many immunological types. Of clinical relevance for man are polio, echo, Coxsackie, and rhinoviruses. HBB and guanidine do not inhibit the multiplication of RNA or DNA viruses belonging to other major groups, nor do they affect the metabolism of the host cell at concentrations at which the reproduction of susceptible viruses is inhibited (106, 108, 113). The actions of HBB and guanidine are similar, but not identical, as revealed for example in their somewhat different virus-inhibitory spectra within the picornavirus group (110–112).

HBB and guanidine have no direct inactivating effect on susceptible viruses (102, 108, 112, 113, 116), nor do they affect virus adsorption or penetration (108, 113, 117–119). They do inhibit the appearance in virus-infected cells of a virus-directed RNA polymerase, an enzyme responsible for the replication of viral RNA (87, 120). Once the enzyme is made, its function does not seem to be impaired by the inhibitors (120, 121). With the inhibition of synthesis of the virus-induced RNA polymerase, no replication of viral RNA can be expected to take place, and this has been experimentally confirmed (117, 122–124). The inhibition of viral RNA synthesis, in turn, leads to inhibition of coat protein synthesis (113, 117), since the viral RNA of picornaviruses acts itself as messenger in

the synthesis of virus-directed proteins. There is, however, evidence that both inhibitors allow coat protein synthesis under experimental conditions, when viral RNA is first permitted to accumulate (125).

The primary sites of action of HBB and guanidine are not yet known. Various possibilities have been considered (118, 126–129), of which we might mention the following two: (*a*) either the inhibitors interfere with the messenger function of viral RNA and thereby inhibit the synthesis of one or several virus-directed proteins, or (*b*) the inhibitors block the morphogenesis of functional viral RNA polymerase from hypothetical precursor peptides.

Of interest are the reports that under certain conditions choline (130), methionine, and valine (130, 131) may interfere with the virus-inhibitory action of guanidine, but probably not with that of HBB (131). The explanation of this finding, apparently not obtained by all investigators (115, 132), awaits further clarification of the mechanism of action of HBB and guanidine.

HBB- or guanidine-resistant mutants of picornaviruses are readily isolated after incubation of infected cell cultures in the presence of these inhibitors (103, 108, 110, 112, 133–135). HBB-resistant mutants are only slightly resistant to guanidine and vice versa (110). This lack of cross resistance is another indication that the sites of action of HBB and guanidine are not identical. In further support of this hypothesis is the demonstration of true synergism in the virus-inhibitory actions of HBB and guanidine (136).

Besides drug-resistant mutants, there have also been isolated HBB-dependent (137, 138) or guanidine-dependent (134, 139, 140) mutants. There is evidence that drug dependence and sensitivity involve opposite drug effects at the same site of action, i.e., that they concern analogous processes in the viral multiplication cycle (118, 124, 141). For instance, guanidine prevents the appearance of viral RNA polymerase in cells infected with drug-sensitive virus, but it is required for the appearance of this enzyme activity in cells infected with guanidine-dependent virus. It has also been demonstrated that in cells infected
the enzyme activity appears whether the compound is present or not (120). Once the enzyme is made, its activity in the cell-free assay system is not affected by guanidine, regardless of whether the enzyme was derived from cells infected with drug-sensitive or drug-dependent virus.

It has been suggested that guanidine dependence and resistance may be "adaptive" phenomena rather than the result of mutation and selection (142). This interpretation does not consider the high mutation rates observed with the HBB and guanidine markers (143).

Infected cells are not protected from ultimate death by HBB or guanidine, although both compounds prevent the acute cytopathic changes observed in picornavirus-infected cells at the time of synthesis of viral RNA and viral coat proteins (144, 145). The ultimate death of infected, treated

cells may be caused by the early virus-induced inhibition of cellular RNA and protein synthesis. These changes are only slightly delayed by guanidine in poliovirus-infected cultures (146, 147).

Guanidine exhibits no significant protective effect in monkeys infected with various poliovirus strains (105, 148). This failure may be caused by the rapid emergence of guanidine-resistant mutant virus (148).

Various structural modifications of HBB have been made and although some derivatives are more active than the parent drug, it remains an open question whether any of these compounds is strikingly more selective than HBB (56, 103, 149–152). Of the two optical isomers of HBB, the D-(−)-isomer is more active than the L-(+) form (153).

Isatinthiosemicarbazones

Isatin-β-thiosemicarbazone and several of its derivatives selectively inhibit the multiplication of poxviruses. The members of this virus group are the largest and most complex animal viruses. They contain DNA. Several poxviruses are of considerable importance in human and animal pathology. The antiviral effect of the thiosemicarbazones was discovered accidentally (154). Subsequent extensive, systematic structure-activity studies yielded compounds of considerable virus selectivity (155–158). Virus inhibitory concentrations of isatin-β-thiosemicarbazone do not markedly affect cell division, cellular DNA, and protein synthesis, or glucose utilization (159–161). The N-methyl derivative also does not inhibit cellular DNA synthesis (162). The first chemotherapeutic trials in man have been reported (163, 164).

In poxvirus-infected cells, the presence of isatinthiosemicarbazone permits the synthesis of viral DNA and of many viral proteins (160, 166–168) but not of all, as revealed by the agar double-diffusion test (168). The missing proteins may be essential for proper virus assembly and maturation; electron micrographs of KB cells infected with vaccinia virus and treated with isatin-β-thiosemicarbazone revealed only immature or abnormal virus particles (166). Viral DNA synthesized in the presence of isatinthiosemicarbazone appears capable of being incorporated into new, functional virus (160, 162).

The primary site of action of the isatinthiosemicarbazones is not known. The parent compound varies markedly in its antiviral activity, depending on the cell type used for virus propagation (168). It is of interest that concentrations of actinomycin, which do not affect multiplication of rabbitpox virus, block the inhibitory action of isatinthiosemicarbazone to a large extent. On the basis of this and other experiments, it has been suggested (168) that the thiosemicarbazone might induce the formation of the true inhibitor. It should be emphasized, however, that cells pretreated with isatinthiosemicarbazone respond to poxvirus infection in the same way as untreated cells (166, 168). The isolation of drug-resistant or drug-dependent mutants of sensitive parent strains has not been report-

ed. Since isatinthiosemicarbazones inhibit the synthesis of only some of the several kinds of poxvirus precursor molecules, it is not surprising that the compound does not prevent cytopathic changes in infected cells (160, 166, 167); such treated cells do not survive and cannot be cloned (161).

Isatin-β-thiosemicarbazone was found over ten years ago to have antiviral activity in vaccinia virus-infected mice (156). These investigations were subsequently extended to several other poxviruses (169), and a number of derivatives of isatinthiosemicarbazone with increased activities were prepared (158, 170, 171). Methisazone (N-methylisatin-β-thiosemicarbazone) was selected for further study, and it protected mice infected intracerebrally with smallpox virus (172). This protection was prophylactic, since the drug was given during the incubation period after virus inoculation.

In a trial of chemoprophylaxis of smallpox with methisazone in man, the compound proved effective (163, 164). 2192 contacts of smallpox patients were treated orally. In this group, six cases of smallpox with two deaths occurred. In contrast, of 2589 untreated contacts, 114 became ill with smallpox, and 20 died. The principal side effect of the treatment was vomiting. It remains to be seen whether the compound may be of use in patients already ill with smallpox (164, 173, 174). There is considerable evidence [see review by Bauer (164)] that methisazone is effective in the treatment of vaccinia gangrenosa and of eczema vaccinatum.

4-Bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone has also been reported to inhibit poxviruses selectively (165). There was no evidence of therapeutic activity by this compound in smallpox patients. However, in a prophylactic trial it provided significant protection (165).

CONCLUSIONS AND PROSPECTS

There is now strong experimental evidence that selective inhibition of animal virus multiplication is feasible. Viral specificity resides in the three-dimensional structure of viral macromolecules, which thus provides virus-specific targets for selective inhibitors. A number of small-molecular virus-selective inhibitors have been found which do not seem to act as antagonists of low-molecular weight precursors essential for both host and virus; presumably they react with specific regions of the macromolecular components of the virus itself, or of virus-directed enzymes. These inhibitors are virus group-specific suggesting that each virus group has certain biochemical features in common. It is theoretically possible, also, that certain structural analogues of metabolites common to virus and host may turn out to be highly virus-selective; in the infected cell there are synthesized virus-coded enzymes which may differ markedly in their substrate affinities from the cellular enzymes. So far, however, this idea has not been experimentally verified.

The primary site of action of virus-selective inhibitors, and the molecu-

lar basis of viral specificity, remain to be defined. It is to be hoped, however, that advances in basic virology will in time permit the deliberate design of virus-selective inhibitors, and reduce reliance on laborious screening and chance.

There are several clinical problems in antiviral chemotherapy. First, treatment of viral diseases requires a rapid diagnosis; this, at present, is often not possible. Sometimes the diagnosis may be made on clinical grounds, but frequently the help of the laboratory is needed. Diagnostic virology is still a young field, and many laboratory tests take considerable time. However, it is reasonable to suppose that techniques will improve and allow speedier reports to the physician (175). Also, since virus-selective inhibitors appear to act group-specifically, treatment may in some cases be intelligently begun before the final virus-typing is completed.

Another problem of considerable clinical importance is the rapid emergence of drug-resistant mutants. The most promising way to deal with this phenomenon is to use combined treatment with compounds lacking cross resistance, and possibly acting synergistically.

The antiviral chemotherapeutic approach is of self-evident importance in the active treatment of virus diseases. However, antiviral chemoprophylaxis is also of considerable medical interest. The control of virus diseases may be achieved primarily by vaccination, but in some types of infection, the large number of serotypes within a virus group, or rapid change in the antigenic makeup of a virus, make vaccination difficult if not impractical. In such situations, chemoprophylaxis would be desirable. Also, for certain diseases in which the occurrence of exposure is often known, chemoprophylaxis may be the procedure of choice.

Antiviral chemotherapy is still in its beginnings. Progress in this field will be of equal importance to basic virology and clinical medicine.

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