

The Behaviour of Recent Isolates of Human Respiratory Coronavirus In Vitro and in Volunteers: Evidence of Heterogeneity Among 229E-Related Strains

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Strains of human coronavirus (HCV) isolated between 1974 and 1976 have been studied in vitro and in volunteers. All strains caused colds in volunteers, and those cultivable in tissue culture (TC) produced significantly more coryza and less sore throat than strains growing only in organ culture (OC). The TC strains were serologically related to 229E, but these isolates produced colds with a frequency and severity that contrasted with the effects of 229E itself. Tests on volunteers' preinfection sera showed that the prevalence of antibody to 229E had increased during the period 1961-1979 and that during 1977-1979 only 11% of subjects had no neutralising antibody against 229E. Susceptibility to the 229E-related isolates PR and TO was associated with low preinfection serum neutralising antibody against the homologous virus, and paired sera frequently showed fourfold or greater antibody rises, most commonly against the homologous strain. Volunteers infected with TO were immune when reinoculated with the same strain approximately 1 year later, but other similar volunteers were at least partly susceptible to infection with a heterologous 229E-related virus after similar time intervals. Although the strains of HCV that were grown in tissue culture were all related to the prototype 229E, they appeared not to be identical with it, and this heterogeneity is probably a significant factor in the epidemiology of HCV infections.

Key words: human coronavirus, 229E, neutralising antibody, common colds, volunteers

INTRODUCTION

Viruses having morphological features that are now considered characteristic of coronaviruses were described in association with human respiratory disease by AI-

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meida and Tyrrell [1967] and McIntosh et al [1967]. Most work on human respiratory coronaviruses (HCV) has been done on the strains 229E, isolated from a specimen collected in 1962 [Hamre and Procknow, 1966] and OC43, isolated during the winter 1965–1966 [McIntosh et al, 1967]. The effects of 229E in volunteers were described by Bradburne et al [1967]. Both strains have been used in a number of seroepidemiological studies, and although antibody to these isolates appears to be relatively common, the viruses are difficult to isolate and outbreaks of infection are seldom identified. It has been suggested that the prevalence of 229E infection fluctuates in 2- or 3-yearly cycles [Monto, 1974], but the pathogenic and epidemiological potentialities of HCVs and the number and interrelationships of their serotypes are still poorly understood. Larson et al [1980], using organ cultures and volunteers, isolated several new HCV strains and confirmed McIntosh's [1974] suggestion that coronaviruses probably cause some 15% of colds in adults. MacLeod and Reed (unpublished results) isolated a further 229E-related strain. The behaviour of these new HCV isolates in tissue culture and in volunteers has now been compared with that of 229E, and some differences have been observed. The clinical responses of the volunteers and measurements of serum neutralising antibody against homologous and heterologous 229E related strains have been used to assess the antigenic heterogeneity among 229E group viruses.

METHODS

Viruses

For studies in volunteers, the seven HCVs isolated by Larson et al [1980] and designated PR, TO, AD, RO, HO, GI, and PA were each used as 1 in 10 dilutions of the original nasal washings obtained from adults with naturally occurring colds. In a few experiments with TO, a similar preparation of nasal washings collected from the first passage of the virus in volunteers (Hu₁) was used as an inoculum for further volunteers. The two strains PR and TO were cultivable in MRC-C cells directly from nasal washings and produced cytopathic effect (cpe) in these cells (TC strains). A third TC strain KI was isolated from a nasal washing collected in 1974 during a study of colds in Antarctica [MacLeod and Reed, unpublished]; it was initially cultivable in human embryo nasal organ cultures and after two passages in organ culture, in MRC-C cells. Passage OC₂ was used for inoculation of volunteers. For experiments in tissue culture, the three TC strains were used after three successive passages at limit dilution in MRC-C cells. Strains AD, RO, HO, GI, and PA were cultivable in human embryo nasal organ cultures but, despite various attempts, not in tissue cultures (OC strains).

The 229E virus isolated in tissue culture was sent to this laboratory in 1965 by Dr. D. Hamre. For inoculation of volunteers, it was passed once in human embryo lung fibroblasts and twice in human embryo nasal or tracheal organ cultures (TC_xOC₂) and then up to six times sequentially in quarantined, isolated volunteers (TC_xOC₂Hu₁ - Hu₆). Between 1972 and 1977, nasal washings of passage levels TC_xOC₂Hu₄, Hu₅, or Hu₆ were used as inocula for volunteers: in 1978 a fresh organ culture passage TC_xOC₂ was used as inoculum. The titre, identity, and purity of inocula used for volunteers were checked in tissue culture. For serological studies, the virus was grown in MRC-C cells and passaged three times at limit dilution. The 229E-related strain LP was isolated from a natural cold in 1965 [Tyrrell et al, 1968], the inoculum

for volunteers, passage Hu₁OC₁Hu₁, had been stored at -70°C since 1966, and for serological studies it was grown in MRC-C cells and used after three serial passages at limit dilution.

Tissue Culture Methods

The continuous cell line MRC-C originally obtained from Dr. A.F. Bradburne was grown in Eagle's basal medium with 10% newborn calf serum and maintained in Leibovitz L15 medium with glutamine 2 mM, 2% foetal bovine serum, gentamicin 50 $\mu\text{g/ml}$ and DEAE dextran 20 $\mu\text{g/ml}$. For virus isolation, culture tubes were incubated on a roller drum at 33°C , and cytopathic effect was read between 3 and 6 days after inoculation. Viral plaques were demonstrated using an overlay containing the same components as the tissue culture maintenance medium with 0.5% agarose; after incubation for 5 days cultures were fixed in formol saline and stained with 1% gentian violet.

Organ Cultures

Human embryo nasal tissue was cultured by a method based on that of Tyrrell and Blamire [1967] using L15 medium with 0.2% bovine albumin. The use of foetal tissue was approved by the Ethical Committee of the Harrow Health District (Clinical Research Centre).

Antisera

Hyperimmune serum against 229E was prepared in rabbits using virions of density 1.18 gm/ml purified on a sucrose gradient by Dr. M. Macnaughton. Initial intramuscular injections of virus emulsified with Freund's complete adjuvant were followed by an intravenous boost without adjuvant. Hyperimmune ascites fluid against LP was prepared in mice by Dr. A.F. Bradburne using the method of Sommerville [1967].

Serological Methods

Neutralising antibody in volunteers' sera was measured by a micromethod using twofold serum dilutions, two replicates per serum, 10–300 TCD₅₀ of virus, and 2×10^4 MRC-C cells per well in microtitre plates. All TC strains except KI, which was insufficiently cytopathic, were used in this test. The serum titre that was read after 5 days of incubation was the highest dilution that completely inhibited development of viral cpe. A fourfold rise in titre between pre- and post-infection sera was considered significant. Neutralisation tests with animal sera were done in test tubes using twofold serum dilutions, three tubes per dilution, and 16–300 TCD₅₀ of virus. Haemagglutination-inhibition antibody against OC43 was measured by the technique of Kaye and Dowdle [1969].

Volunteers

Volunteers aged 18–50 came from all areas of the UK and were housed in isolation at the Common Cold Unit as previously described [Tyrrell, 1963]. Some of the subjects made several visits to the Unit at approximately yearly intervals. Symptoms were assessed and scored daily [Beare and Reed, 1977] and after 3 days of quarantine, inocula containing coronavirus or control fluids were given as nasal drops, and the subjects were observed for a further 6 days. Saline-inoculated control

subjects were included in all experiments, and all clinical evaluations were done double blind. Nasal washings for virus isolation were collected on at least two occasions between the second and fifth days after inoculation, depending on the timing of symptoms. Representative virus isolates were checked for neutralisation with a hyperimmune rabbit serum prepared against 229E virus. Sera were collected from volunteers before and about 3 weeks after virus inoculation. The experiments were approved by the Ethical Committee of the Harrow Health District (Clinical Research Centre).

RESULTS

Plaque Morphology

The strains 229E, PR, TO, and KI formed plaques in MRC-C cells (Fig. 1). PR plaques were indistinguishable from 229E, and those of TO were probably slightly

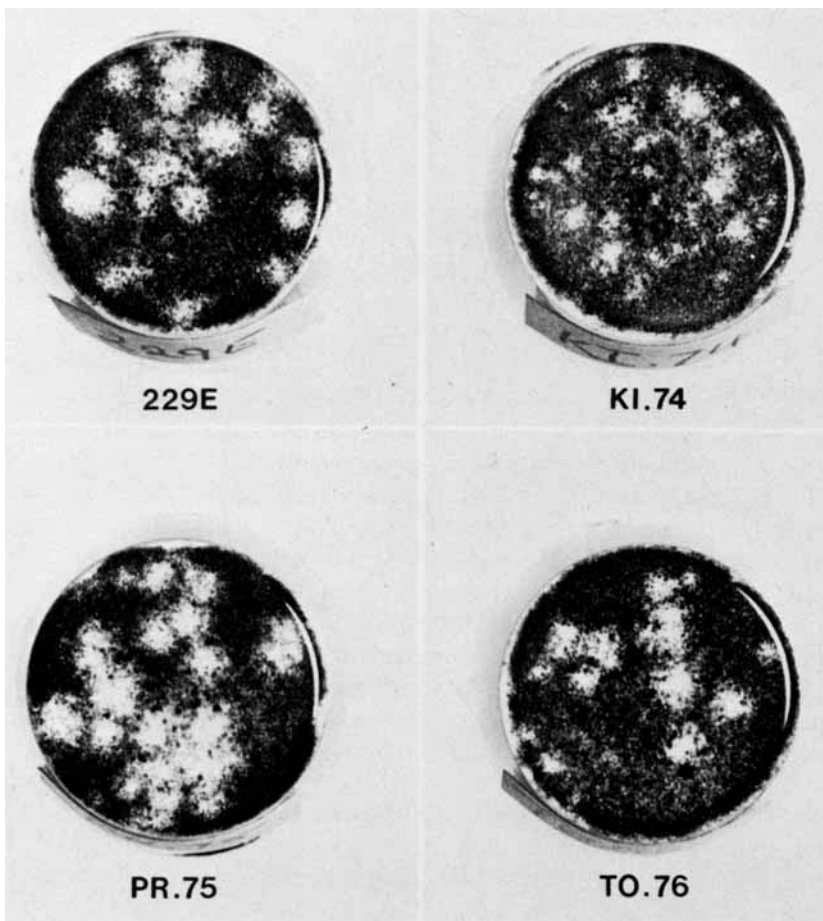


Fig. 1. Plaques produced by HCV TC strains 229E, KI, PR, and TO in MRC-C cells in wells 15-mm in diameter cut from plastic multiwell culture plates. Cultures were fixed in formol saline 5 days after inoculation and stained with gentian violet.

smaller, but all plaques had indefinite edges, making measurements difficult. KI produced small, indistinct plaques and was also less strongly cytopathic than the other strains in MRC-C cells in both test tubes and microtitre plates.

Neutralisation of TC Strains by Hyperimmune Sera

The TC strains 229E, TO, PR, KI, and LP were all neutralised by a hyperimmune rabbit serum against 229E and an ascites fluid from mice immunised with LP. However, the end point titres of these two serological reagents against the five virus strains showed differences that were consistent in each experiment. Every experiment with the LP immune fluid or the 229E antiserum included the homologous and one or more of the heterologous viruses. Using a standard dose of the five virus strains, the range of titres obtained with the LP fluid was greater than the range obtained with the 229E serum (Table I): The mean titre of LP fluid against its homologous antigen LP was almost 16-fold higher than against the heterologous strain PR, whereas with 229E antiserum the difference between homologous (229E) and heterologous (PR) titres was about 4-fold. Thus, both the antiserum and the immune fluid detected differences between TC isolates; the greater ability of the LP immune fluid to do this may reflect both the nature of the immunising antigen and the method of immunisation.

Effects of Recent Isolates in Volunteers

Most of the coronaviruses isolated in 1974–1976 and tested between 1976 and 1979, produced relatively frequent and severe colds (Table II). The highest mean clinical score was produced in 1977 by PA virus, an isolate that was not cytopathic for MRC-C cells. Two TC strains, PR and TO, produced moderately high mean scores, and virus was reisolated directly in tissue culture from nasal washings of the majority of subjects given those strains. Paired sera from about half the volunteers given PR or TO showed fourfold rises in neutralising antibody titre against the homologous virus. There was good correlation in individual volunteers between development of symptoms, virus reisolation, and antibody rises. Eight (42%) of the 19 subjects given PR and 9 (60%) of the 15 given TO were found to have no neutralising antibody (titre < 1:2) against the homologous virus in their preinoculation sera. Nasal washings from most of the volunteers who developed symptoms after inoculation of the strains AD, RO, HO, GI, and PA were tested in MRC-C cells, but no cpe developed. It was, therefore, not possible to use the homologous strain as antigen when testing sera from the volunteers who received these viruses. However, sera from these volunteers showed no significant rises in neutralising antibody titre using 229E or PR antigens or in HI antibody using OC43 antigen.

TABLE I. Neutralisation of TC Strains in MRC-C Cells by Hyperimmune Serum or Ascites Fluid

Serum or ascites fluid against	Mean neutralisation titre (\log_2) of serum or ascites fluid against 16–300 TCD ₅₀ of these viruses				
	229E	LP	KI	PR	TO
229E	9.71 (5) ^a	8.79 (2)	8.13 (1)	7.95 (2)	8.46 (2)
LP	9.87 (4)	9.91 (3)	8.13 (2)	6.13 (2)	6.46 (3)

^aFigures in parentheses give the number of observations from which the means were derived.

TABLE II. Responses of Volunteers to Inoculation of Recent Isolates of HCV

Strain (and year of origin)	Years of testing	Number of subjects	Clinical response		Proportion giving virus isolation	Proportion showing antibody rise	Mean antibody titre pre- and postinoculation ^b
			Total No. of colds ^a	Mean clinical score			
KI(74)	1978	19	12(1)	15.0	14/16	ND ^e	ND
PR(75)	1978-79	19	11(5)	19.6	13/19	9/19 ^b	1.72-6.39
TO(75)	1977	15	13(4)	27.6	13/15	8/15 ^b	1.67-6.96
PA(76)	1977	16	13(10)	31.7	0/16	0/7 ^c , 0/6 ^d	ND
AD(74)	1976-77	11	8(1)	12.4	0/6	0/8, ^c 0/6 ^d	ND
RO(75)	1976-78	16	12(3)	14.6	0/10	0/5 ^d	ND
HO(75)	1979	15	4(0)	9.7	ND	ND	ND
GI(75)	1978	9	7(0)	19.0	0/5	0/5 ^c , 0/5 ^d	ND

^aNumbers of subjects graded as having moderate or severe colds are given in parentheses.

^bGeometric mean of reciprocals of neutralising antibody titres using homologous antigen.

^cNeutralisation tests using 229E and PR antigens.

^dHAI test using OC43 antigen.

^eND = Not done.

The symptoms produced by the TC strains TO, PR, and KI were compared with those produced by the OC strains AD, RO, PA, GI, and HO (Table III). TC strains produced coryza significantly more often and sore throat less often than the OC strains. This finding corresponds with observations of natural infection [Hendley et al, 1972].

The effect of TO in volunteers was studied in 5 successive years—1977–1981 (Table IV). In each year groups of 8 to 21 subjects received either a 10^{-1} dilution of the original nasal washing TO (in 1977, 1978, and 1979) or of passage Hu₁ of the same virus (in 1979, 1980, and 1981). No volunteers who had received a coronavirus inoculum on any earlier visit to the Unit were included in these groups. The symptoms experienced by these subjects, as indicated by the mean clinical scores, decreased in successive years and the proportion of volunteers having preinoculation neutralising antibody to TO increased over the same period, as did the mean preinoculation antibody titres.

229E in Volunteers

The effects produced by the newer HCV isolates were in marked contrast with those produced between 1972 and 1978 by several different inocula of the prototype 229E (Table V). The colds produced by 229E during this period were relatively few and mild. In 1972 it was thought possible that prolonged storage and low-titred inocula of 229E might have been responsible. The preparation TC_xOC₂Hu₄ was, therefore, passaged twice in volunteers producing inocula TC_xOC₂Hu₅ and TC_xOC₂Hu₆; similarly, the preparation TC_xOC₁, stored at -70°C for 11 years, was passaged in organ culture to give a higher titred inoculum TC_xOC₂. These procedures did not increase the severity of the infections produced in volunteers. However, it was noted that most of the volunteers possessed serum neutralising antibody against 229E. Another 229E-related TC strain, LP, was also tested in volunteers during 1977–1979 (Table V). This inoculum had been stored at -70°C under the same conditions as 229E for 11 years (1977) or 13 years (1979), but its clinical effects were very much more striking. Preinoculation antibody titres against LP were lower than against 229E.

TABLE III. Symptoms Associated With Colds Produced in Volunteers by TC and OC Strains of Coronavirus

	TC strains ^a	OC strain ^b	Significance of difference ^c
Number of colds assessed	40	67	
Incubation period in days (mean and range)	2.6(1–3.5)	3.1(1–3.5)	
Coryza	25 (62.5%)	26 (38.8%)	P < 0.05
Sore throat	15 (37.5%)	49 (73.1%)	P < 0.001
Cough	15 (37.5%)	32 (47.8%)	Not significant
Pyrexia	8 (20%)	21 (31.3%)	Not significant

^aTO, PR, KI.

^bAD, RO, GI, HO, PA.

^cChi-squared test.

TABLE IV. Effects in 1977-1981 of Inoculation of HCV TO in Groups of Volunteers Who Had Received No Previous Experimental Inoculation of Coronavirus

Years of testing	Number of subjects	Clinical response		Proportion giving virus isolation	Proportion showing antibody rise	Mean antibody titre pre- and post inoculation ^b
		Total no. of colds ^a	Mean clinical score			
1977	15	13(4)	27.6	13/15	8/15	1.67-6.96
1978	8	4(2)	16.4	5/8	5/8	2.03-6.49
1979	21	16(2)	14.4	16/21	4/20	2.52-5.16
1980	11	6(2)	14.4	8/11	1/10	3.56-7.13
1981	15	7(2)	12.4	ND ^c	ND	ND

^aNumber of subjects graded as having moderate or severe colds are given in parentheses.

^bGeometric means of reciprocals of antibody titres.

^cND = not done.

TABLE V. Responses of Volunteers to Inoculation With Various Preparations of 229E Virus, 1972-1978, and LP Virus

Virus	Passage history	Dose per volunteer (TCD ₅₀)	Year of study	Number of subjects	Clinical response			Proportion giving virus isolation	Proportion showing rise of antibody	Proportion antibody free ^b	Mean antibody titre pre- and postinoculation ^c
					Total no. of colds ^a	Mean clinical score	Mean				
229E	TC _x OC ₂ Hu ₄ and Hu ₅	10 ^{1.25}	1972	22	4(1)	4.0		7/22	2/20	4/22	4.27-6.77
229E	TC _x OC ₂ Hu ₄ and Hu ₅	10 ^{1.5} or 10 ^{1.25}									
229E	TC _x OC ₂ Hu ₆	10 ^{1.5}	1975-77	24	7(0)	3.0		12/24	5/21	3/21	4.01-7.46
229E	TC _x OC ₂ Hu ₆	>10 ^{2.5}	1977	5	3(0)	7.8		2/5	0/4	1/4	2.06-3.83
229E	TC _x OC ₂	10 ^{4.2}	1978	12	6(0)	6.5		7/12	1/12	1/12	4.95-6.93
LP	Hu ₁ OC ₁ Hu ₁	NR ^d	1977-79	18	15(6)	24.9		16/18	10/18	7/18	2.27-9.02

^aNumber of subjects graded as having moderate or severe colds are given in parentheses.^bProportion of subjects having neutralising antibody titre less than 1 in 2 in preinoculation serum.^cGeometric mean of reciprocals of antibody titres.^dNR = not recorded.

TABLE VI. Prevalence of Neutralising Antibody Against HCV 229E in Random Adult Sera, 1961-1979

Date of collection of sera	Number of subjects	Mean antibody titre ^a	Proportion (and percentage) with antibody titre < 1:2
1961-62	30	3.1	8/30(26.7%) ^b
1966-67	36	3.2	8/36(22.2%)
1971-72	30	4.4	5/30(16.7%)
1977-79	63	5.0	7/63(11.1%) ^b

^aGeometric mean of reciprocal of antibody titre.

^bThe difference between these proportions is significant at the 5% level (chi-squared test, $P < 0.05$).

Because of the mild effects of 229E in volunteers and the presence of preinoculation antibody in most subjects, a retrospective study was done to assess whether the immune status of the population had changed since 1962 when the virus was first isolated [Hamre and Procknow, 1966]. Four groups of 30 to 62 sera were randomly selected from among preinoculation sera collected from volunteers in varying months of all years between 1961 and 1979. All sera had been stored at -20°C under standard conditions. The subjects providing them were aged between 18 and 50, and the mean ages of all the groups lay between 30.0 and 34.0 years. Between 1961 and 1962 and 1977 and 1979 the proportion of sera showing no detectable neutralising antibody against 229E declined, and the mean antibody titre of the groups rose (Table VI). A similar survey for OC43 HI antibody over the same period revealed fluctuating levels rather than a steady rise.

Antibody Responses Against Heterologous 229E Group Viruses

The clinical effects of the 229E-related strains were more varied than might have been expected from the results of neutralisation tests with hyperimmune sera. To investigate further the serological diversity of the strains paired, pre- and postinfection sera from the 18 volunteers who developed colds and shed virus after inoculation of strain TO in 1977 or 1978 were tested by the microneutralisation method against the homologous antigen TO and the heterologous strains 229E and PR (Fig. 2). Mean titres were higher against 229E than against TO or PR (229E: 1 in 5.2 preinfection and 1 in 13.7 postinfection; TO: 1 in 1.6 and 1 in 7.7; PR: 1 in 1.4 and 1 in 5.3), but fourfold rises were commoner against the homologous antigen (229E, 6 rises; TO, 12 rises; PR, 10 rises). This difference between 229E and TO, although not achieving statistical significance (χ^2 test, $0.05 < P < 0.1$), again suggests that serological differences exist between 229E and the two newer strains.

Volunteers' Immunity to Reinoculation

In a few instances during 1977-1979, it was possible to reinoculate individual subjects at approximately yearly intervals using the same or different HCV strains. Six subjects who were inoculated with strain TO in 1977 received the same virus

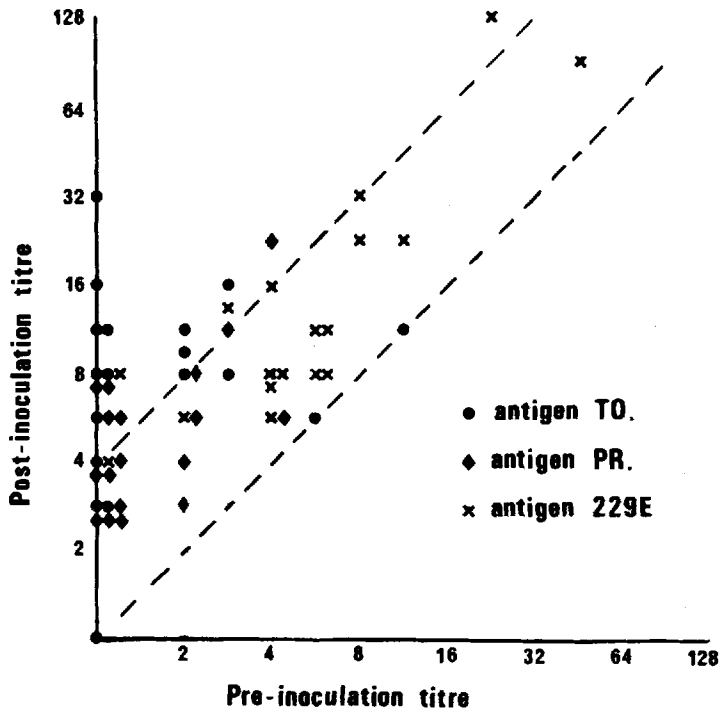


Fig. 2. Neutralisation titres obtained with paired sera from 18 volunteers who were infected with HCV strain TO. The sera were tested against the homologous antigen TO and the heterologous antigens 229E and PR.

again 8–12 months later. After the first inoculation, all had colds and the mean clinical score was high; virus shedding was easily detectable in all six subjects, and five of them developed antibody rises. In the second season, all six subjects appeared completely immune to reinoculation; the mean clinical score was 1.3, and no virus shedding or significant antibody rises were detected. Eight other subjects developed mild or moderate cold symptoms and shed virus after an inoculation of 229E, LP, KI, TO, or DP and were each reinoculated 8 to 14 months later with a heterologous, 229E-related TC strain, either LP, KI, TO, or DP. In contrast to the immunity found after homologous reinoculation, five of the eight subjects receiving a heterologous strain on the second occasion developed cold symptoms (mean clinical score 10.5) and five shed virus. Similarly, four other volunteers who developed colds after inoculation of the OC strain PA, which is related to 229E by ELISA (Macnaughton, 1981b), were challenged 11–13 months later with the TC strain KI. Two of the four subjects developed colds after challenge (mean clinical score 12.8) and three shed virus. Although the number of observations on heterologous challenge is small and the possible effects of natural intercurrent infections cannot be documented, these results suggest that infection with a 229E group virus confers only partial immunity against subsequent infection with another 229E-related strain, whereas full immunity to homologous reinfection lasts at least a year.

DISCUSSION

Macnaughton et al [1981a,b] using the ELISA technique on paired sera from inoculated volunteers found that all the isolates described in the present study were related either to 229E or to OC43, although the strains PA and AD that were designated OC because they were not cultivable in MRC-C cells nevertheless fell into the 229E antigenic group. Thus, there are clearly at least two major serogroups of HCV. The antigenic complexity of OC43-related strains is already well documented: McIntosh [1974] studied a number of OC isolates and concluded that they were variably related to each other, to OC43, to MHV, and perhaps to 229E. All TC strains so far examined are related to 229E and have been thought to form a relatively homogenous group, but few of them have been studied in detail, although Bradburne [1970] noted minor differences between 229E and strain LP. The present study suggests that 229E-related TC strains, like OC43-related strains are not all identical.

The high incidence of HCV infection in adults [Larson et al, 1980] suggests either that immunity to the two major types 229E and OC43 in the individual and the community is transient or that antigenic variants exist, which are not completely cross-protective, or perhaps that both these possibilities apply. Monto [1974] suggested that 229E virus becomes prevalent in the community every 2 to 3 year, and with this hypothesis of 2- to 3-year "cycling" of 229E in mind, our inability between 1972 and 1978 to obtain more than very few minor infections with 229E virus in volunteers seemed surprising, but it became clear that most subjects had preinoculation neutralising antibody to 229E, and that the prevalence of this antibody had apparently steadily increased between 1961 and 1979. The new isolates all produced colds in volunteers, although some strains appeared more pathogenic than others. For the TC strains, increased pathogenicity or virulence for volunteers was associated with low preinoculation neutralising antibody titres against the homologous virus, but for the OC strains it was not possible to establish whether the strain-specific immune status of the recipients was a reason for the apparent variations in strain virulence. Rechallenge experiments in volunteers suggested that the various 229E-related strains were only partly cross-protective, although immunity to reinoculation of the same strain lasted at least a year. Thus, the present results suggest that the existence of serological variants of HCV probably has an important bearing on the frequency and severity of infections in the individual and in the community. The diversity of 229E-related variants or subtypes may be sufficient to allow a rapid succession of infections, and on the present evidence this seems more likely than repeated reinfections with a single serotype.

The neutralisation test probably involves interactions with the viral surface, and ELISA also measures antibody against surface projections, the reactivity having been shown to be mainly against the large, glycosylated, spike-associated polypeptide rather than the membrane- or ribonucleoprotein-associated polypeptides [Macnaughton et al, 1981a]. Both the neutralisation and the ELISA test show the relationship of 229E to other TC strains, but although microneutralisation appeared less sensitive than ELISA [Kraaijeveld et al, 1980] and gave very low titres with volunteers' sera, it nevertheless showed differences between 229E and the other TC strains that were not revealed by ELISA and that appeared to correspond with their clinical effects. The neutralisation test may, therefore, detect both 229E group-specific and subtype-specific components; and modifications of this technique, such as the kinetic neutral-

isation test [Bradburne, 1970] or neutralisation in organ culture [Darbyshire et al, 1979], although cumbersome, might help in differentiating strains. Although the general biochemical structure of coronaviruses is now becoming clearly established [Siddell et al, 1982], it is not yet known how many antigenic determinants are associated with the surface polypeptides of HCV nor to what extent the polypeptides of different HCV strains vary. Studies with monoclonal antibodies against HCV have not yet been reported but may prove rewarding in defining the antigenic diversity of the strains. Similarly, genome analysis aided by the use of restriction enzymes may also help to establish the inter-relationships of these viruses.

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