three lymphocyte function tests. It would seem that the cellular immune response to an unrelated organism is not impaired in patients with herpetic infection and that the defects in macrophage migration inhibition activity and lymphocyte cytotoxicity are confined to *H. hominis* type 1 antigen. Long-term sequential studies will be pursued to test the hypothesis that impaired macrophage migration inhibition and lymphocyte cytotoxicity responses in the presence of a positive lymphocyte transformation and antibody reactions are responsible for recurrent herpetic infection.

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PRELIMINARY COMMUNICATIONS

Trimethoprim Resistance determined by R Factors

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Summary

R factors conferring a high level of resistance to trimethoprim have been identified in one strain of *Escherichia coli* and one of *Klebsiella aerogenes*, both isolated from infected urines.

Introduction

Trimethoprim (TMP) became available for general use in medicine three years ago. It is a synthetic substance which inhibits the enzyme dihydrofolate reductase, necessary for the essential synthesis by bacteria of folinates, and it acts synergistically with sulphonamides, whose antibacterial effect is on an earlier stage in the same synthetic pathway (Hitchings, 1969). Therapeutic preparations of trimethoprim are always put up in combination with a sulphonamide (Su). The rationale for this is to make use of their synergistic antibacterial effects and at the same time prevent the emergence of mutant bacteria resistant to either drug (Bushby, 1969). The combination of trimethoprim and sulphonamide has proved useful in many kinds of bacterial

infection, and notably in combating invasion of the urinary tract by Enterobacteriaceae (Grüneberg and Kolbe, 1969; Reeves et al., 1969). Sulphonamide resistance is not uncommon in bacteria causing urinary infection (Williams and Leigh, 1966; Grüneberg, 1971; Shaw et al., 1972). The incidence of resistance to trimethoprim is low; it is currently found, in our experience, in about 1% of urinary infections with *Escherichia coli* (Grüneberg, 1971).

We report here the isolation from infected urines of bacteria highly resistant to both trimethoprim and sulphonamide in which the resistances were determined by transmissible R factors.

Materials and Methods

Bacteria.—Strains of Enterobacteriaceae, highly resistant to trimethoprim, were collected by Dr. E. Joan Stokes, Miss Pamela Waterworth, and R.N.G. from clinical material sent to the diagnostic laboratories at University College Hospital. Antibiotic sensitivity tests were made in the first instance by a disc diffusion method in which the size of zone given with the test strain is compared with that given using a standard sensitive strain (Stokes, 1968). Sulphonamide and trimethoprim sensitivity tests were performed separately on a medium free of inhibitors. There were five cultures of *E. coli* whose minimal inhibitory concentration (M.I.C.) for trimethoprim was 16 µg/ml or more, all resistant also to sulphonamide and one of them to tetracycline. There were three cultures of *Klebsiella* spp. and one of *Enterobacter aerogenes*, all resistant to ampicillin, trimethoprim (M.I.C. 32 µg/ml or more), and sulphonamide, and with various patterns of resistance to streptomycin (S), tetracycline (T), chloramphenicol (C), nalidixic acid, and nitrofurantoin. One further trimethoprim-sulphonamide-resistant *E. coli* was isolated subsequently (see below).

Minimal Inhibitory Concentrations.—Small inocula (broth cultures diluted sufficiently to give isolated colonies) were streaked on plates lacking trimethoprim-sulphonamide inhibitor (Waterworth, 1969) and containing serial dilutions of drug. The M.I.C. was taken as the lowest concentration which prevented visible growth.

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Resistance Transfer.—The 10 trimethoprim-resistant cultures were examined for R factors by M.P.F. and N.D. Each was grown in mixed broth culture with a nalidixic-acid-resistant *E. coli* recipient and the mixtures were plated on a selective medium (not containing sulphonamide-trimethoprim inhibitor) incorporating nalidixic acid 50 µg/ml and trimethoprim 5 µg/ml. The recipient was *E. coli* K12 J62-1—non-lactose fermenting, nutritionally exacting, and sensitive to antibacterials other than nalidixic acid. Neither donor nor recipient grew on the selective medium. Colonies developing from the mixed cultures were purified, identified as *E. coli* K12 by their biochemical and nutritional characteristics, and tested for sensitivity to a range of antibacterial drugs including trimethoprim. In some experiments selection was made with sulphonamide or other drug to which the donor was resistant instead of trimethoprim. Where the K12 recipient had acquired resistance during growth in mixed culture it was tested for the ability to transfer that resistance to other distinguishable sublines of *E. coli* K12 and in one instance to *Salmonella typhimurium* WM 0024. When an R factor was thus detected it was transferred into a line of K12 carrying the F factor (Hayes, 1953) so that its *fi* (fertility inhibition) character (Watanabe and Fukasawa, 1962) could be examined. *Fi*+ R factors repress the production of F pili by *F*+ *E. coli*, while *fi*− R factors do not. They are therefore distinguishable by testing the sensitivity of *R*+*F*+ cultures to *F*-specific phages, whose site of adsorption is to the F pili.

Results

Transfer of Resistance.—Of the nine primary strains one *E. coli* (EC1) (of four collected by R.N.G.) and one *Kl. aerogenes* (KA1) (of five strains received from Dr. Stokes and Miss Waterworth) transferred trimethoprim resistance to *E. coli* K12. *E. coli* EC2, tested subsequently, also transferred resistance to *E. coli* K12 and to *Salm. typhimurium*. The R factors from *E. coli* EC1 and EC2 determined resistance only to trimethoprim and sulphonamide and were *fi*−. The factor from *Klebsiella* KA1 conferred resistance to streptomycin, tetracycline, and chloramphenicol as well as to trimethoprim and sulphonamide; it was *fi*+. K12 strains to which resistance was transferred from EC1 and EC2 were always resistant to both trimethoprim and sulphonamide no matter which of these drugs was used for selection. With the R factor from KA1 all five resistance markers (S, T, C, Su, TMP) were transferred together when either trimethoprim or sulphonamide or chloramphenicol was used for selection (Table I).

TABLE I—Trimethoprim-resistant Bacteria

Strain	M.I.C. for TMP (µg/ml)	Other Drug Resistance	Resistance Transferable to K12
<i>E. coli</i>			
EC1	>1,000	Su	TMP Su
EC2	>1,000	Su	TMP Su
EC3	64	Su	—
EC4	32	Su	—
EC5	256	Su	Su
C14	64	Su T	Su
<i>Kl. aerogenes</i> KA1	>1,000	Su A S T C	TMP Su S T C
<i>Kl. aerogenes</i> KA2	>1,000	Su A S C	—
<i>Klebsiella</i> species K19	32	Su A C	—
<i>Ent. aerogenes</i> K10	128	Su A S	—

Frequencies of Transfer.—The frequency of transfer from the naturally-occurring resistant strains to *E. coli* K12 or *Salm. typhimurium* during overnight incubation of mixtures was approximately 10^{-7} per introduced donor cell. Between lines of K12, incubated in mixed culture for one hour, the R factors from EC1 and EC2 transferred at a frequency of 2×10^{-3} per donor cell, and that from KA1 at 10^{-2} .

Levels of R-factor-determined Resistance.—The M.I.C.s of trimethoprim and of sulphonamide of K12 lines to which the R factors were transferred were over 1,000 µg/ml.

ORIGIN OF R+ STRAINS

EC1 was isolated from the urine of a woman aged 76 who had been in a geriatric ward at University College Hospital for six months. In August 1971 an infection of her urinary tract caused by *Kl. aerogenes* sensitive to trimethoprim and sulphonamide had been treated with this combination for 14 days. Four days after completing treatment another urinary infection was found, this one caused by *E. coli* 018 (EC1), which was resistant to trimethoprim and sulphonamide. This infection was eradicated with a course of nitrofurantoin. Both infections were probably endogenous since there had been no instrumentation of the urinary tract and both the organisms were subsequently recovered from the patient's rectal flora.

Examinations of the rectal flora of all the other patients in the ward were performed by picking 10 lactose-fermenting colonies from a rectal swab culture from each and testing these for resistance to trimethoprim. One resistant colony was found among the 270 strains thus examined. This derived from a woman aged 86. She had been treated with trimethoprim-sulphonamide in January. A month later she had developed an infection of the urinary tract with *Kl. aerogenes*, which was resistant to trimethoprim. This had responded to treatment. She had subsequently received a variety of courses of chemotherapy for unrelated conditions, including a further course of trimethoprim-sulphonamide. The single trimethoprim-resistant colony (EC2) proved to be *E. coli* 018, the same type as EC1 above. Examination of ward records showed that in a three-month period six courses of trimethoprim-sulphonamide had been given to patients in that ward.

KA1 was isolated from the urine of an 80-year-old man who had been admitted to the hospital in October 1971. He had not previously received trimethoprim-sulphonamide but had been treated with a variety of other antibacterial agents.

PREVALENCE OF TRIMETHOPRIM RESISTANCE

Among 280 cultures of enteric bacteria isolated from cases of urinary infection in general practice 2.1% were resistant to trimethoprim and 20.7% to sulphonamide. For 309 hospital cultures these figures were 7.4% and 27.5% respectively. The higher frequency of resistance to trimethoprim among the hospital strains is largely accounted for by the greater number of urinary infections in hospital caused by *Klebsiella* spp., in which trimethoprim resistance is common (33%) (Table II).

TABLE II—Frequency of Trimethoprim and Sulphonamide Resistance in Urinary Pathogens in General Practice and in Hospital in 1971

Organism	No. of Strains in General Practice			No. of Strains in Hospital		
	TMP Resistance	Su Resistance	Total	TMP Resistance	Su Resistance	Total
<i>E. coli</i>	2	46	229	3	55	208
<i>Klebsiella</i> spp. ..	1	3	6	15	22	45
Other	3	9	45	5	8	56
Total	6	58	280	23	85	309

Discussion

Resistance factors (R factors) of bacteria were discovered in Japan in 1959. They are transmissible plasmids, or DNA elements, evidently similar to bacterial viruses in some respects (Anderson, 1968). They are not only harmless to their bacterial hosts but confer on them resistance to various environmental hazards, in particular to antibacterial drugs used in medicine. Most of the drugs to which they give resistance are naturally-occurring antibiotics, and the way R factors act is in many cases by providing their host with genetic information for the synthesis of drug-inactivating enzymes—for example, penicillinases

and chloramphenicol acetylase (for review see Davies, and Rownd 1972). Such enzymes may possibly have evolved to protect bacteria against naturally occurring antibiotics. But the R factors first identified, as well as many recognized later all over the world, give resistance to sulphonamides as well as to antibiotics.

The mechanism of R-factor-mediated sulphonamide resistance is not known. Can it have evolved since the first use of sulphonamides in the 1930s? It seems unlikely. More probably a biochemical mechanism protective against sulphonamides already existed, evolved in response to unknown pressures, and was selected and encouraged to spread by the medical use of sulphonamides. Previously known R factors carrying genes for sulphonamide resistance do not, in our experience, confer resistance to trimethoprim. Lebek and Wiedmer (1971) and Lebek (personal communication) found, however, that some such R factors conferred a low level of trimethoprim resistance. The R factors we describe here give resistance to both drugs (as well as, in one case, to unrelated antibiotics). We do not know whether separate genes control resistance to the two synthetic drugs or whether a single biochemical mechanism gives resistance to both.

Resistance to trimethoprim was found in 1% of strains of *E. coli* isolated in 1966 from urinary infections in general practice two years before the drug became generally available for medical use. The prevalence of trimethoprim resistance in clinical isolates of *E. coli* remains at about this level in both hospital and domiciliary practice (Grüneberg, 1971). The prevalence of trimethoprim resistance in *Klebsiella* spp. in hospital is higher (33%) than in *E. coli*. It is not known how much of the trimethoprim resistance at present found in enteric bacteria is R-factor determined.

The widespread use of trimethoprim may encourage the spread of such R factors, but up until now no such spread has become apparent in *E. coli*. R factors giving resistance, especially

to streptomycin, the tetracyclines, and sulphonamides, are common in the normal bacteria of the human intestine (Datta, 1969; Datta *et al.*, 1971). All these drugs have been in use for over 20 years. Infections caused by bacteria resistant to them occur, and the proportion of R+ urinary infections outside hospital has increased, though not appreciably so, during the past decade (Shaw *et al.*, 1972; Gillespie *et al.*, 1971). What will happen in respect of trimethoprim-resistant R factors can at present only be a matter for speculation. We hope that clinical bacteriologists will monitor and report any changes which may occur in the usefulness of this valuable drug.

Salm. typhimurium WM 0024 was kindly supplied by Dr. J. R. Walton.

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MEDICAL MEMORANDA

Haemolytic Anaemia associated with Antibodies to Tolbutamide and Phenacetin

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Several drugs are known to give rise to a positive direct Coombs test with or without haemolytic anaemia (Pirofsky, 1969; Robinson and Faodi, 1969; Stefanini and Johnson, 1970; Henry *et al.*, 1971). There are at least three types of drug-dependent positive direct Coombs tests: (1) the stibophen type, in which the drug combines with antibody and the complex is adsorbed non-specifically on to the red cells and fixes complement; (2) the penicillin type, where the drug is readily

attached to red cells, which are then either directly agglutinated by anti-penicillin antibody or, if the antibody is weak, shown by a positive indirect Coombs test; and (3) the Aldomet (methyldopa) type, in which methyldopa either alters the red cell membrane or affects the antibody-forming tissues so that an autoantibody is produced which has a specificity within the rhesus blood group system. The drug takes no part in the serological reaction. Robinson and Faodi (1969) distinguished a fourth or cephalothin type; cephalothin, however, probably operates by mechanisms 1 and 2.

We here report the case of a patient with an immune haemolytic anaemia associated with tolbutamide, a sulphonylurea compound, and phenacetin.

Case Report

The patient, an Italian woman aged 58, presented with a history of lassitude, dyspnoea, headaches, and weakness of the legs for four weeks. Routine blood tests on 9 July 1971 showed haemoglobin 7.4 g/100 ml, P.C.V. 22%, M.C.H.C. 33%, and reticulocytes 30.5%. A blood film showed pronounced anisocytosis, poikilocytosis, and polychromasia, with normal white cell morphology. Haemolytic anaemia was suspected. She was admitted to hospital on 11 July and on examination appeared anaemic.

There was no evidence of bruising, lymphadenopathy, splenomegaly, or hepatomegaly, and the finger-nails were normal. Her pulse rate was 90/min and regular, blood pressure 130/90, and there were no other appreciable clinical features. A full blood investigation on 14 July showed: haemoglobin 7.8 g/100 ml;

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