observations are perhaps worthy of mention. Firstly, whereas with crystalline vitamin B₁₂ very little further improvement can be expected after six months' parenteral therapy (Ungley, 1949), in most of our cases a steady improvement was noticed over the first two years of treatment. Secondly, on parenteral therapy, according to Denny-Brown (1959), it is "unlikely that signs that have been present for months can be reversed." In the present series, however, although the symptoms in 8 of the 11 cases were of six months' duration or more, some improvement in the initial signs occurred in all except one case (No. 71).

Finally, much has been heard during the past few years about the significance of vitamin-B₁₂ levels in the serum on vitamin-B₁₂-peptide therapy. It has been widely assumed that because low serum-vitamin-B₁₂ levels were found to be the gloomy heralds of haematological and neurological disaster when patients were receiving intrinsic-factor preparations, a similar interpretation of low levels was also justifiable on vitamin-B₁₂-peptide therapy.

Our preparations, however, are not intrinsic-factor preparations, and the assumption, therefore, that low serum levels have the same significance on both forms of therapy is still unproved. We believe, in fact (Mooney and Heathcote, 1961b; Heathcote and Mooney, 1961), that serum-vitamin-B₁₂ levels have little practical value in vitamin-B₁₂-peptide therapy, and in this connexion it is noteworthy that one patient (Case

67) has achieved complete recovery not only from all signs and symptoms of anaemia but also from nerve degeneration, although his serum-vitamin-B₁₂ level has never exceeded 55 µg./ml.

Summary

Eleven cases of subacute combined degeneration of the spinal cord were treated orally with vitamin-B₁₂-peptide preparations for periods up to four years.

The average dose ranged from 30 to 300 µg. (equivalent) of vitamin B₁₂ a day.

In seven cases an excellent response was obtained and all patients are leading normal lives.

Three patients showed fairly good responses, and one—in whom the disease had been present for 14 years—showed almost no improvement.

It is a pleasure to acknowledge our gratitude to Dr. E. T. Baker Bates and Dr. G. M. S. Ryan for generous access to a number of their cases.

REFERENCES

- Denny-Brown, D. (1959). In Cecil and Loeb's *Text Book of Medicine*, 10th ed., p. 1505. Saunders, Philadelphia.
 Heathcote, J. G., and Mooney, F. S. (1958a). *Lancet*, **1**, 982.
 ——— (1958b). *J. Pharm. Pharmacol.*, **10**, 593.
 ——— (1961). *Vitamin B₁₂ and Intrinsic Factor. 2nd European Symposium*, p. 540.
 Mooney, F. S., and Heathcote, J. G. (1960). *Lancet*, **2**, 291.
 ——— (1961a). *Brit. med. J.*, **1**, 232.
 ——— (1961b). *Ibid.*, **2**, 53.
 Ungley, C. C. (1949). *Brain*, **72**, 382.

SCREENING OF 12 GLIOMATA AGAINST CHEMOTHERAPEUTIC AGENTS IN VITRO

BY

DOROTHY M. EASTY, Ph.D.

JOHN A. H. WYLIE, M.A., M.D., D.Phil.

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London

Ambrose and his colleagues (1962) devised a method whereby the sensitivity of human tumour cells obtained at biopsy or operation, and grown in tissue culture, could be tested quantitatively against chemotherapeutic substances. As these are primary cultures, and have presumably changed little compared with cells *in vivo*, it was felt that information obtained in this way might be of value to the clinician in his choice of drugs for the treatment of individual cases.

Using the methods described in detail below, we have attempted to cultivate many types of tumours, and whereas we have had scant success with most carcinomata and sarcomata, our experience with gliomata, comprising astrocytomata, ependymomata, and medulloblastomata, has been almost invariably successful. The results obtained in the testing of 12 gliomata and a culture of HeLa cells are reported here.

Methods and Materials

The specimens examined were all obtained from Atkinson Morley's Hospital or the Hospital for Sick Children at Great Ormond Street. At operation the tumours are placed in either a dry sterile screw-capped bottle or into Parker's TC medium 199 and packed in ice in a vacuum flask. They are brought to the Chester Beatty Research Institute and arrive within the hour. Immediately on arrival the specimens are chopped up manually with fine scissors and, being very soft, fragment easily. The "mush" so obtained is transferred to a 0.06% solution of commercial trypsin ("difco") in Parker's TC medium 199 and agitated in a magnetic

stirrer for 30 minutes at 37° C. Thereafter the coarser particles in the suspension are allowed to settle and the supernatant fluid is pipetted off and centrifuged for five minutes at 1,500 r.p.m. The supernatant from this procedure, which contains the trypsin, is removed and the centrifuged cells are resuspended in the final medium, which consists of 20% of the patient's serum + 80% Evans N.C.T.C. medium 109, to which folic acid (0.4 mg./litre) and insulin (438 mg./litre) have been added. Repeated washings of the cells were found to give less satisfactory results, and it is felt that the trypsin inhibitors in the serum incorporated in the medium are sufficient to neutralize the traces of trypsin remaining after centrifugation.

The suspension thus achieved is dispensed in 0.3-ml. quantities into Ambrose tubes (Ambrose *et al.*, 1962) and incubated at 37° C. in a gas phase of 5% CO₂ in air. A good monolayer of cells on the base of the tubes has usually formed in 48 hours and the medium is then changed, giving a clear fluid through which observations with an inverted microscope can be made. The monolayer is almost always dense enough to allow counting to be performed with the Chalkley eyepiece (Curtis, 1960). The drugs selected for administration are then added according to the method already described (Ambrose *et al.*, 1962), the same volume—0.08 ml.—of Hanks's saline being added to the control tubes.

The drugs chosen for this series were: melphalan (*p*-di-(2-chloroethyl)amino-L-phenylalanine), thiotepe (triethylene thiophosphoramide), chlorambucil (*p*-di-(2-chloroethyl)aminophenylbutyric acid), mannitol busul-

phan ("myleran") (1,6-(dimethanesulphonyl)-1,6-deoxymannitol), and methotrexate (*N*-[*N*-*p*-(2,4-diaminopterid-6-ylmethyl)-*N*-methylaminobenzoyl]-L-glutamic acid).

Methotrexate was found to be uniformly lacking in cytotoxic effect in this system, and has been abandoned in favour of mannitol busulphan, which, weight for weight, is less toxic *in vivo* than the other alkylating agents used (Haddow *et al.*, 1958). Mannitol busulphan was used in the concentration range 2.5×10^{-5} to 2.5×10^{-3} g./ml., whereas melphalan, chlorambucil, and thiotepa were exhibited at 1/10 of these concentrations.

Counts of the cells adhering to the base of the Ambrose tubes were made daily for five days, including the initial count. After this period the cells in the control tubes tended to become granular and detached, so the results became unreliable. In any event the pattern of effects of the alkylating agents was clear after this time and did not alter appreciably when it was found possible to prolong the culture.

Results

Each chemotherapeutic compound was added at three concentrations, the second and third being ten times the serial dilutions of the first, giving a hundredfold range of concentrations.

Counts were made of the control and treated cultures daily for periods varying from three to six days. On each the LD_{50} * value of each compound was determined. These values, expressed in g./ml. of culture medium, are given in Table I for nine astrocytomata, one probable astrocytoma, one ependymoma, and one medulloblastoma. Also included in the Table for comparison are results obtained using a culture of HeLa human epidermoid carcinoma cells (Glaxo Line 39).

Discussion

The method of obtaining the LD_{50} value is demonstrated in Fig. 1. Chalkley counts are plotted against the three concentrations of the test drug (melphalan in this case). The counts in the control tubes on successive days are indicated by C_1 , C_2 , and so on. The initial count in this case was 25, and the LD_{50} taken as 13. The concentrations of melphalan needed to cause a drop in cell count to this value on three successive days are shown in the graph.

In the five experiments in which methotrexate was used it was not found to have any cytotoxic activity at the concentrations used, although in one case it did seem to have a cytostatic effect—that is, the prevention of an increase in cell number, although the control counts were increasing—at a concentration of 2.5×10^{-4} g./ml. Since this drug is an antimetabolite, a folic acid antagonist, this lack of activity may be due to the composition of the growth medium used. In view of the small quantities of material available for testing, it was decided to replace methotrexate with mannitol busulphan (Haddow *et al.*, 1958) as a test compound.

It can be seen from Table I that chlorambucil, and to a less extent melphalan, are quick-acting compounds. That is, the cytotoxic effect of the compound can be seen after one or two days' treatment, and after this the LD_{50} value decreases very slowly, if at all. In the case of thiotepa, however, there appears to be a time

TABLE I.— LD_{50} Values Expressed in 10^{-4} g./ml.

Tumour	Days of Treatment with Drug	Melphalan	Chlorambucil	Thiotepa	Methotrexate	Mannitol Busulphan
Medulloblastoma (glioma 17)	1 2 3 4	14 2.5 2.5 1.6	1.3 1.1 1.1 1.0	>25 >25 10 1.4 0.25	>25 >25 >25 >25	
Astrocytoma grade 4 (glioma 19)	1 2 3 6	10 1.2 0.9 0.25	ca. 13 9 7 7	>25 15 2.5 0.15	>25 >25 >25 >25	
Astrocytoma grade 3 (glioma 22)	1 2 3 4	18 6 0.2 0.19	18 14 12 ca. 1.4	>25 16 ca. 1 0.18	>25 >25 >25 >25	
Astrocytoma grade 3 (glioma 24)	1 2 3 4	15 15 14 14	16 12 10 10	20 14 1.7 1.8	>25 >25 >25 >25	
Glioma 25	1 2 3 4	14 12 11 10	14 12 11 11	>25 2 ca. 0.16 ca. 0.14	>25 >25 >25 >25	
Astrocytoma (glioma 27)	1 2 3 4	16 13 2.2 1.8	>25 19 15 14	>25 18 9 2.5		>25 23 15 14
Astrocytoma (glioma 28)	1 2 3	15 11 10	18 14 14	25 11 2		130 16 15
Astrocytoma grade 3 (glioma 29)	1 2 3 4	13 11 1.5 1.3	23 13 9 4	>25 16 9 0.8		19 17 14 <14
Astrocytoma (glioma 31)	1 2 3 4	25 2.3 2.3 2.1	>25 >25 21 21	>25 14 11 1.0		210 150 70 14
Ependymoma grade 3 (glioma 32)	1 4 5	14 1.9 1.7	20 19 15	>25 0.21 0.18		>250 100 18
Astrocytoma grade 3 (glioma 33)	1 2 3 4	6 2 2 2	14 11 11 10	25 17 2.1 1.0		150 60 25 12
Astrocytoma grade 3 (glioma 35)	1 2 3 4 7	7 1.5 1.7 1.7 1.7	20 13 13 13 15	>25 14 2.5 1.4 0.23		140 140 2.5 1.9 2.0
HeLa (Glaxo Line 39)	1 2 3 4	17 2.5 1.8 1.2	14 2 2 2	16 13 2.1 1.3		>25 >25 21 7.5

FIG. 1.—Melphalan on glioma 28 (astrocytoma).

lag before the effect of the drug can be seen. Then after one or two days' treatment the LD_{50} decreases sharply and continues to decrease over the four days of treatment. Mannitol busulphan also appears to have a time-lag before its effects become evident (see Fig. 2). These observations are in agreement with the fact that thiotepa requires some time *in vivo* to be converted to the active form which is an effective anti-tumour agent, and that mannitol busulphan acts more slowly anyway (T. A. Connors, personal communication).

*The LD_{50} value is defined as the concentration of a test compound which causes the lysis of 50% of the cells originally present in a culture.

At the end of Table I are included the results obtained using a monolayer culture of HeLa cells, this particular line being kindly supplied by Glaxo Laboratories. In Table II the LD_{50} values obtained in this experiment after four days are compared with the average values obtained with the same compounds against five gliomata (four astrocytomata and one ependymoma) after the same length of time.

FIG. 2.—Cytotoxic effect of chemotherapeutic agents on an astrocytoma (glioma 28).

TABLE II.— LD_{50} Values in 10^{-6} g./ml.

	Melphalan	Chlorambucil	Thiotepa	Mannitol Busulphan
5 gliomata ..	1.8	14	1.1	13 (mean of 3 readings)
HeLa	1.2	2	1.3	7.5

It can be seen that in the case of chlorambucil and mannitol busulphan the HeLa cells have a lower LD_{50} than the five gliomata, and much the same value with melphalan and thiotepa. This seems to indicate that cell lines are not necessarily more resistant than primary cultures in this type of assay.

From Table I it can be seen that the most cytotoxic compound after four days' treatment is thiotepa in eight cases, melphalan in two cases, and almost equal values for these two drugs in the remaining two cases. Since the culture of normal human tissues is difficult to maintain for the required period, and we have only succeeded in keeping normal brain growing for three days, it was felt that a more useful value would be obtained by comparing this cytotoxic concentration *in vitro* with the dose causing a certain recognizable effect on the blood when used clinically. It was decided (D. A. G. Galton, personal communication) to use for this value the single adult dose of these compounds, which in clinical practice causes approximately a 95% drop in neutrophil count. The values used are given in Table III.

TABLE III.—Single Dose of a Compound Causing a 95% Drop in Neutrophil Count (Intravenous Injection)

Chlorambucil	250 mg.
Melphalan	100 "
Mannitol busulphan	24,000 "
Thiotepa (intramuscular dose reducing total white blood count to 3,000)	40 "

It can be seen that mannitol busulphan has a value approximately 100 times greater than that of chlorambucil, which has the next highest tolerated dose. The tissue culture therapeutic index is then defined as the ratio of this dose in g./kg. body weight (assuming an adult weight of 70 kg.) divided by the cytotoxic LD_{50} dose in tissue culture after a treatment lasting four days

or longer. For instance, in the case of the penultimate astrocytoma in Table I, the most cytotoxic compound is thiotepa, but the calculated values of the tissue culture therapeutic indices are as follows: mannitol busulphan 2,800, melphalan 70, thiotepa 60, and chlorambucil 36—that is, mannitol busulphan has a much more favourable index according to this test than the other compounds.

In most of the cultures which have been studied the cells have been maintained—that is to say that no change in cell number in the controls has been observed. But in five cases, all astrocytomata, and in the case of the HeLa cells, mitosis obviously took place, and the Chalkley count increased. In three astrocytomata and the HeLa cells there were indications that there is a concentration of the test drugs, considerably lower than the LD_{50} value, which is not toxic to the cells already present, but which will prevent mitosis from taking place. With rapidly growing cultures it may be possible to determine more accurately this "cytostatic" dose and to calculate a cytostatic therapeutic index.

It has been mentioned that the LD_{50} values of the drugs for the gliomata and HeLa cells are not very different, neither are they for the different types of glioma. So far as the astrocytomata are concerned these findings have mainly academic interest, as the tumours are usually solitary and do not as a rule metastasize to other parts of the central nervous system. The position with ependymomata and medulloblastomata is very different. These tumours are prone to seed across the cerebrospinal fluid and can be transported elsewhere in the central nervous system. For this reason our findings may have some practical applications. By the intrathecal administration of an active drug it may be possible to delay or prevent transthecal metastasis. In this respect, too, our findings are fortunate in that thiotepa is the most cytotoxic drug of the range used and mannitol busulphan always has the most favourable tissue culture therapeutic index. Both these drugs are directly soluble in aqueous solution, in contrast to melphalan and chlorambucil, shown here to be less effective, which have to be dissolved in ethanolic HCL and ethanol respectively, as well as requiring to be buffered with propylene glycol. These solvents alone would almost certainly preclude the use of melphalan and chlorambucil in the theca. Experiments in rabbits and dogs are currently being conducted by Dr. H. J. G. Bloom to determine the toxicity of thiotepa and mannitol busulphan when administered intrathecally.

Summary

A series of nine astrocytomata, a probable astrocytoma, an ependymoma, and a medulloblastoma were cultivated *in vitro* and their sensitivity to a variety of chemotherapeutic drugs was compared with a similarly cultivated strain of HeLa cells. No startling or consistent differences in sensitivity were observed among the cells grown, although marked variation was found to pertain to the various drugs administered. Methotrexate was quite inactive in this system. Melphalan and chlorambucil acted rapidly, but were in the end less toxic than thiotepa. The tissue culture therapeutic index has been defined and has been found invariably to favour mannitol busulphan on account of the low haemotoxicity of this drug *in vivo*.

The application of the findings to the treatment of transthecal metastasizing brain tumours is discussed.

ADDENDUM.—Since the preparation of this paper we have included in our battery of chemotherapeutic agents "epodyl" (I.C.I.) and vincalcukoblastine, on the advice of Mr. L. Walsh, of Atkinson Morley's Hospital, and Mr. K. Till, at Great Ormond Street, respectively. In the five gliomata tested (four astrocytomata and one ependymoma) vincalcukoblastine has proved very cytotoxic but occasionally variable in its effect in cells *in vitro*; this is in accordance with the behaviour of the drug *in vivo* (Dr. G. Hamilton Fairley, personal communication). In contrast, epodyl has proved more cytotoxic in most cases than mannitol busulphan and has often an even higher chemotherapeutic index.

We are grateful to Mr. Wylie McKissock, of Atkinson Morley's Hospital, and to Mr. Kenneth Till, of the Hospital for Sick Children, Great Ormond Street, London, for supplying us with brain-tumour material and to Dr. H. J. G. Bloom, of the Royal Marsden Hospital, for organizing this supply. We also wish to thank Dr. E. J. Ambrose, Professor F. Bergel, F.R.S., and Professor A. Haddow, F.R.S., for their interest in this work and for their helpful advice and criticism, and also Miss Vivienne O'Connor for technical assistance.

This investigation has been supported by grants to this Institute from the British Empire Cancer Campaign, the Medical Research Council, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

REFERENCES

- Ambrose, E. J., Andrews, R. D., Easty, D. M., Field, E. O., and Wylie, J. A. H. (1962). *Lancet*, **1**, 24.
Curtis, A. S. G. (1960). *Med. biol. Illus.*, **10**, 261.
Haddow, A., Timmis, G. M., and Brown, S. S. (1958). *Nature (Lond.)*, **182**, 1164.

Medical Memoranda

Erythema Nodosum in Association with Pustular Ringworm

Erythema nodosum is generally regarded as part of a clinical syndrome, the cause of which undoubtedly varies with the climate, geographic location, and the prevalence of any one predisposing disease.

In this country, where Willan, in 1808, was the first to describe the condition of erythema nodosum, tuberculosis was thought to be the prevalent predisposing disease. This was so at the turn of this century, but it was later noted by Forman (1936) and many others that streptococcal infection was by far the most frequent cause of this condition. It was not until 1941 that King, in the U.S.A., made the observation that 111 of 141 patients with erythema nodosum showed hilar-gland enlargement on x-ray examination of the chest. This observation was confirmed by van Beek and Haex (1943), Löfgren (1946), and others, and shown to be part of a generalized sarcoidosis.

In a study of 70 cases of erythema nodosum in England, Vesey and Wilkinson (1959) showed 45.6% to be of proved or presumptive streptococcal origin, 5.6% were tuberculous, 35.7% accompanied sarcoidosis, and 13% were of mixed or unknown origin, of which none were fungal.

The present view is that erythema nodosum occurs most often as a sequel to haemolytic streptococcus infection of the upper respiratory tract, less frequently in tuberculosis, sarcoidosis, and leprosy, and rarely in syphilis, lymphogranuloma inguinale, coccidiomycosis,

and blastomycosis. In the endemic areas of the southern states of the U.S.A. primary infection of incomers by coccidiomycosis is often asymptomatic or manifest only by what would pass for influenza (Dickson, 1937), such manifestations often being accompanied by erythema nodosum, from which most patients make an uncomplicated recovery (Dickson, 1938a, 1938b). Smith *et al.* (1946), in a review of 1,351 cases of coccidiomycosis, found 4.6% to have erythema nodosum. In an epidemic of North American blastomycosis involving 10 patients, Smith *et al.* (1955) found three cases of erythema nodosum, an association not previously described. In these deep fungal infections erythema nodosum does occur, but is rarely found in association with the superficial dermatomycoses. With the decline in the incidence of the superficial mycoses, the present case of a young boy with such a syndrome is recorded.

CASE REPORT

The patient was a boy aged 7 years 7 months. Some two weeks prior to examination he had developed "red itchy patches" on the forehead and thigh, which increased in size and became rough and scaly. These were soon followed by a "huge red lump" on the back of his head. He kept a white mouse, which he allowed to run over his head. (The animal was disposed of following the clinical diagnosis, and comparative mycological study was not obtained.)

On examination an inflamed boggy swelling of pustular ringworm exuding seropus, 4 cm. in diameter, was seen over the occipito-parietal area of the scalp. On the forehead and thigh were two discrete circinate areas of scaly ringworm. There was a bilateral cervical adenitis. A faint, diffuse pale erythema was noted over the lower limbs. The temperature was 101° F. (38.3° C.).

He was admitted to hospital for investigation and treatment. The day after the initial examination he was found to have developed a marked erythema nodosum over both shins.

Investigations.—Mycological culture revealed *Trichophyton mentagrophytes*. Haemoglobin was 12.3 g./100 ml.; P.C.V. 39%; M.C.H.C. 31.5%; E.S.R. 48 mm. after 1st hour (Westergren). Direct swab from pustular ringworm showed a heavy growth of *Staphylococcus albus* (coagulase-negative). Throat swab showed no haemolytic streptococci. X-ray examination of the chest revealed no abnormality. Mantoux intracutaneous test (1 in 10,000) was negative.

Clinical Progress.—On treatment with systemic griseofulvin and topical measures the ringworm lesions steadily healed, with an associated regression of the erythema nodosum and fall in the E.S.R. Recovery was uneventful.

COMMENT

Mycological investigation of similar uncomplicated cases have revealed that a large proportion of mice sold in the West of Scotland as pets or as laboratory animals are infected with *Tr. mentagrophytes*.

J. F. FERGUSON SMITH, V.R.D., M.B., Ch.B.,
Senior Registrar, Department of Dermatology,
Western Infirmary, Glasgow.

REFERENCES

- Beek, C. van, and Haex, A. J. C. (1943). *Acta med. scand.*, **113**, 125.
Dickson, E. C. (1937). *Arch. intern. Med.*, **59**, 1029.
— (1938a). *J. Amer. med. Ass.*, **111**, 1362.
— (1938b). *Amer. Rev. Tuberc.*, **38**, 722.
Forman, L. (1936). *Brit. J. Derm.*, **48**, 123.
King, D. S. (1941). *Amer. J. Roentgenol.*, **45**, 505.
Löfgren, S. (1946). *Acta med. scand.*, Suppl. 174.
Smith, C. E. *et al.* (1946). *Amer. J. publ. Hlth*, **36**, 1394.
Smith, J. G., *et al.* (1955). *J. Amer. med. Ass.*, **158**, 641.
Vesey, C. M. R., and Wilkinson, D. S. (1959). *Brit. J. Derm.*, **71**, 139.
Willan, R. (1808). *On Cutaneous Diseases*. Johason, London.