Table 2. Effect of KCl on the activities of dihydrofolate reductases from strains of *P. vinckei* and *P. berghei* resistant to and sensitive to pyrimethamine. The assays were performed as described (*3*) in the presence of buffer [0.015*M* tris(hydroxymethyl)aminomethane-hydrochloride (tris-HCl) (*p*H 7.0; 37°C)] and the indicated concentrations of KCl, which were added to the buffer. In each case the activity in 0.015*M* tris-HCl was set equal to 100 for ease of comparison.

| KCl (*M*) | *P. vinckei* | | *P. berghei* | |
|---|---|---|---|---|
| | Sensitive | Resistant | Sensitive | Resistant |
| None | 100 | 100 | 100 | 100 |
| 0.15 | 156 | 96 | 300 | 133 |
| 0.21 | 116 | 92 | 280 | 119 |
| 0.51 | 114 | 74 | 214 | 75 |

zyme from the strain selected as a product of the mixed infection (the resistant *P. berghei*) should be readily discernable. The data support this assumption; major differences were found between the dihydrofolate reductases of the sensitive and resistant *P. berghei* strains (Tables 1 and 2). The enzyme from the resistant strain had a ninefold higher $V_{max}$, a tenfold higher $K_m$ value for dihydrofolate, and a 20-fold increase in the 50 percent inhibitory concentration for pyrimethamine, as compared to the enzyme from the sensitive *P. berghei* (Table 1). Also, the resistant *P. berghei* enzyme was stimulated less effectively by KCl than the enzyme from the resistant strain (Table 2), and the inhibition of the enzyme by pyrimethamine was unaffected by prior incubation with dihydrofolate (Table 1).

The possibility that extracts of the resistant *P. berghei* contained a mixture of separate molecules of the enzyme from the resistant *P. vinckei* and the sensitive *P. berghei* was investigated. Inspection of curves of percentage inhibition plotted against the logarithm of pyrimethamine concentrations for the enzymes from the latter two strains revealed that there is a range of pyrimethamine concentrations ($0.8 \times 10^{-8}$ to $4.0 \times 10^{-8}M$) in which the sensitive *P. berghei* enzyme is completely inhibited and the resistant *P. vinckei* is unaffected. The 50 percent inhibitory concentration of pyrimethamine found for the enzyme from the resistant *P. berghei* ($1 \times 10^{-8}M$) fell within this range of concentrations; thus, if it were a mixture, it would have to contain equal amounts of the two enzymes, and a graph of the percentage inhibition versus the logarithm of pyrimethamine concentration would be expected to show a broad "plateau" at this range of concentrations. Such a plateau was in fact observed when a prepared mixture (1 : 1) of the enzyme from the resistant *P. vinckei* and from the sensitive *P. berghei* was tested for inhibition by pyrimethamine. However, the curve for the resistant *P. berghei* did not show this plateau. A straight line was obtained with the same slope as in the case of the other three enzymes; thus it was concluded that the dihydrofolate reductase was not a mixture of the enzyme from the "donor" and "recipient" strains.

The properties of the dihydrofolate reductase from the resistant *P. berghei* were intermediate in character between those of the resistant *P. vinckei* and the sensitive *P. berghei*. The marked differences among the enzymes from the various strains suggest that the protein structures of these enzymes are not identical. In the resistant *P. vinckei* this is presumably due to a mutant gene or genes coding for an altered dihydrofolate reductase, selected during the development of resistance to pyrimethamine. It seems likely that only part of the gene or genes responsible for coding of the enzyme of the donor strain would be transferred to the recipient. Hotchkiss and Evans (*8*) and Sirotnak *et al.* (*9*) have reported on multiple gene sites involved in drug resistance in bacteria, in which different degrees of resistance could be transferred in transformation experiments. Also, altered dihydrofolate reductases are present in mutant strains of antifolate-resistant bacteria (*8, 10*). The experiments reported here, and previously (*2*), indicate that these phenomena can occur in plasmodia. It may be difficult to determine the mechanism by which the transfer occurred in view of the absence of techniques for genetic analysis in plasmodia. However, by arranging conditions to allow for biological filtration, and by detailed analysis of the genetic marker chosen, our studies support the contention that transfer of genetic material among plasmodia is possible.

ROBERT FERONE
MARY O'SHEA
*Wellcome Research Laboratories, Burroughs Wellcome & Co. Inc., Tuckahoe, New York 10707*
MEIR YOELI
*Department of Preventive Medicine, New York University School of Medicine, New York 10016*

### References and Notes

1. J. Greenberg and H. L. Trembley, *J. Parasitol.* **40**, 667 (1954); B. M. Honigberg, *J. Protozool.* **15**, 223 (1968); P. J. Walker, in *Infectious Blood Diseases of Man and Animals*, D. Weinman and M. Ristic, Eds. (Academic Press, New York, 1968), vol. 1, p. 367.
2. M. Yoeli, R. S. Upmanis, H. Most, *Parasitology* **59**, 429 (1969).
3. R. Ferone, J. J. Burchall, G. H. Hitchings, *Mol. Pharmacol.* **5**, 49 (1969).
4. R. Ferone, *Fed. Proc.* **28**, 847 (1969).
5. ———, *J. Biol. Chem.*, in press.
6. F. M. Huennekens, *Biochemistry* **2**, 151 (1963).
7. H. Lineweaver and D. Burke, *J. Amer. Chem. Soc.* **56**, 658 (1934).
8. R. Hotchkiss and A. H. Evans, *Fed. Proc.* **19**, 912 (1960).
9. F. M. Sirotnak, G. J. Donati, D. J. Hutchison, *J. Biol. Chem.* **239**, 4298 (1964).
10. R. Berberich and M. Levinthal, *Bacteriol. Proc.* **1969**, 54 (1969).
11. We thank J. Sklarsh for his technical assistance. The work on malaria carried out by M.Y. is under the sponsorship of the Commission on Malaria, A.F.E.B., and is supported in part by the U.S. Office of the Surgeon General, Department of the Army.

22 October 1969

## Mixed Lymphocyte Reactions and Tissue Transplantation Tolerance

Abstract. *The induction of tolerance of Lewis histocompatibility antigens in BN rats inoculated at birth with BN/Lewis $F_1$ hybrid bone marrow cells, as revealed by the prolonged survival of Lewis skin grafts, is accompanied by markedly decreased reactivity in the mixed lymphocyte interaction. Blood lymphocytes from animals inoculated with lymph node cell suspensions also display diminished proliferative reactivity to hybrid BN/Lewis cells in the interaction. However, these recipients are not tolerant of Lewis skin grafts. Blood lymphocytes from BN rats inoculated neonatally with Lewis thymocytes fail to display any level of unresponsiveness in vitro, and such animals are not tolerant of Lewis skin grafts. The results suggest that in rats skin and marrow cells have histocompatibility antigens that are absent or poorly expressed on lymph node cells and thymocytes.*

In rats, bone marrow cells constitute the only effective means by which neonatal recipients can be rendered tolerant of Ag-B incompatible skin grafts (*1*), that is, skin grafts that differ from their host at the major histocompatibility locus of the species (*2*). By comparison, cells derived from the spleen and lymph nodes are poor in inducing tolerance of these grafts and thymocytes are ineffective (*3*). Nonetheless, there is evidence that rats inoculated at birth with Ag-B incompatible lymph node or splenic cells are persistent cellular chimeras. Blood leukocytes from these animals will immunize isologous recipients against skin homografts from the donor strain (*4*); and a high pro-

portion of these rats will succumb to a graft-versus-host reaction if specifically sensitized lymphocytes from the donor strain are administered to them in numbers that ordinarily would have no effect on normal recipients (*4*). Situations where cell chimerism persists in the absence of tolerance of skin homografts have also been described for mice (*5*), cattle (*6*), and man (*7*).

Peripheral blood lymphocytes derived from rats made tolerant by inoculation at birth with Ag-B incompatible bone marrow cells have been shown to be specifically unreactive in the mixed lymphocyte interaction (MLI) (*8*). This inability to be stimulated in vitro by cells bearing the tolerance-inducing antigens is thought to reflect the state of induced specific immunological tolerance of skin homografts in vivo. It therefore seemed important to determine whether the poor results in inducing tolerance of skin homografts in rats with lymph node cells or thymocytes was associated with a change in reactivity of cells from such animals in the MLI. The absence of any significant proliferative activity by lymphocytes from these animals when exposed in culture to cells bearing the tolerance-inducing antigens, concomitant with the failure of these animals to accept test skin homografts, might imply that tolerance of cells derived from one tissue may not necessarily extend to cells of another tissue of the same genetic origin.

Rats of the isogenic BN (Ag-$B^3$) strain were inoculated within 24 hours after birth with either $30 \times 10^6$ bone marrow, lymph node, or thymus cells obtained from young adult BN/Lewis (Ag-$B^3$/Ag-$B^1$) $F_1$ hybrids (*9*). Hybrid donors were used to avoid lethal graft-versus-host reactions. Proliferative reactivity of the peripheral blood lymphocytes from these recipients was tested in the MLI when the animals were 8 weeks old. One million blood lymphocytes derived from each of them were mixed in culture with 1 million BN/Lewis $F_1$ hybrid lymphocytes according to procedures described in detail by Wilson (*10*). The use of this experimental design provides a unidirectional response, since previous studies have shown that proliferating cells in parental-$F_1$ cultures are almost exclusively of parental origin (*8*). The activity obtained in these mixtures was compared with the activity of cells from normal BN rats mixed with $F_1$ hybrid cells. All cultures were conducted in triplicate in a volume of 1 ml of culture medium with 10 percent fresh rat serum. In vitro activity was assessed on day 5 and day 6 by adding tritiated thymidine ([$^3$H]TdR, 0.1 ml containing 0.25 $\mu$c; 6.7 c/mmole) 16 hours before harvesting the cultures. Incorporation of [$^3$H]TdR into the acid-insoluble (trichloroacetic acid) fraction was determined by liquid scintillation spectrometry (*10*).

All neonatally inoculated animals, as well as a panel of normal adult BN hosts, were grafted with Lewis skin within 1 week after they provided cells for the assay in vitro. Survival of the test grafts was scored daily after the initial 8-day inspection (*11*).

Finally, leukocytes from some of the animals inoculated with lymph node cells were retested for in vitro activity 6 months after graft rejection to determine whether the tolerant state, as assessed by this assay, had changed.

The results of the comparison of proliferative activity of cells in the MLI and survival of test skin homografts on subjects inoculated at birth with homologous cells of different origin are presented in Table 1. As expected, the majority of animals inoculated with bone marrow cells proved to be highly tolerant of test skin homografts (such grafts survived for more than 50 days) and all of them were comparatively unreactive in the MLI.

The groups inoculated with lymph node cells or with thymocytes, on the other hand, displayed some interesting differences. None of them proved to be even partially tolerant in terms of skin graft survival. The median survival time (MST) of test grafts on these animals was not different from that of normal first-set homografts on untreated animals (Table 1). However, whereas blood lymphocytes from most of the rats inoculated with lymph node cells proved to be comparatively nonreactive in the MLI, lymphocytes from rats that had been inoculated at birth with thymocytes gave proliferative responses similar to those of cells from normal animals.

Table 1. Mixed leukocyte responses and skin homograft reactions of BN rats inoculated at birth with Lewis cells of different origin.

| Cells injected | Recipients (No.) | Distribution of mixed leukocyte responses (% of controls)* | | Graft survival (days) | | |
|---|---|---|---|---|---|---|
| | | (0–40) | (>40) | Highly tolerant (>50) | Tolerant (14–50) | Not tolerant (<14) |
| Bone marrow | 16 | 16 (15.9 ± 6.1)† | 0 | 11 | 4 | 1 |
| Lymph node | 15 | 12 (25.3 ± 5.8) | 3 (60.6 ± 13.6) | 0 | 0 | 15‡ |
| Thymus | 14 | 0 | 14 (112.1 ± 19.8) | 0 | 0 | 14§ |

* Percentages were calculated from the "peak" response of each animal. † Mean percent of control response (± 2 S.E.). ‡ MST, 8.5 ± 1.2 days. § MST, 8.0 ± 0.3 days. (The MST of Lewis grafts on BN hosts is 8.1 ± 0.4 days.)

Finally, although some change in the in vitro activity was observed when the MLI was repeated on ten lymph node cell recipients 6 months after they had acutely rejected Lewis skin grafts, their postgrafting responses were still significantly reduced and in all but two animals remained less than 40 percent of control values.

These experiments show that BN rats inoculated at birth with BN/Lewis lymph node cells may reject Lewis skin grafts with the vigor and promptitude of normal animals, even though their blood lymphocytes are significantly less reactive to Lewis cells in culture. The basis for this paradox is unknown. One possibility is that bone marrow and skin cells share an antigen(s) that is poorly represented on lymph node cells so that tolerance induced with bone marrow cells extends to skin and is reflected by the lack of proliferative activity to leukocyte-borne antigens in the MLI; tolerance induced with lymph node cells, on the other hand, extends only to the antigens on lymphocytes.

A complete absence in lymphocytes of one or more of the important Ag-B antigenic specificities present on skin cells does not seem very likely since neonatal treatment with high dosages ($\geq 30 \times 10^6$) of lymph node cells occasionally does result in rats' accepting skin grafts indefinitely (*12*). This could, however, be due to the presence of marrow-derived cells in the suspensions employed.

It is also unlikely that bone marrow cells and skin differ from lymph node cells with respect to some non-Ag-B determined antigen(s) since lymph node cells (and thymocytes) are effective in inducing high degrees of tolerance of subsequent skin grafts when donor and host are Ag-B compatible (*1*), unless

the presence of Ag-B antigens interferes with the ability of node cells to induce tolerance of other than Ag-B factors. The MLI occurs only between Ag-B incompatible cells (*13*).

A deficiency of one or more of the Ag-B specificities present in marrow, lymph node, and skin cells seems a more likely explanation for the tolerogenic shortcoming of thymocytes, since lymphocytes from rats inoculated at birth with these cells are fully reactive in vitro. Furthermore, high degrees of tolerance of skin grafts have never been induced by inoculation of these cells (*3*). It may be pertinent that in mice the content of H-2 isoantigen in thymus cells is significantly reduced in the presence of thymus-specific antigens (*14*). Another, although less likely, possibility is that thymocytes fail to reach or persist in the appropriate organs of the host for tolerance to be induced.

Although all available evidence suggests that skin grafts are more exacting in their histocompatibility requirements than homografts of other organs—kidney, liver, heart, and so forth (*15*)—it remains to be determined whether the poor ability of lymph node cells and thymocytes to induce tolerance when tested with skin grafts applies to homografts of other organs as well. This question is currently under investigation.

WILLYS K. SILVERS
DAVID M. LUBAROFF
DARCY B. WILSON, DIANNE FOX
*Immunobiology Research Unit, Departments of Medical Genetics and Pathology, University of Pennsylvania School of Medicine, Philadelphia 19104*

### References and Notes

1. W. K. Silvers and R. E. Billingham, *Transplantation* **8**, 167 (1969).
2. W. L. Elkins and J. Palm, *Ann. N.Y. Acad. Sci.* **129**, 573 (1966).
3. R. E. Billingham and W. K. Silvers, in *The Thymus*, V. Defendi, Ed. (Wistar Institute Press, Philadelphia, 1964), pp. 41–48.
4. R. E. Billingham, V. Defendi, W. K. Silvers, D. Steinmuller, *J. Nat. Cancer Inst.* **28**, 365 (1962).
5. N. L. Warner, L. Herzenberg, L. J. Cole, W. E. Davis, *Nature* **205**, 1077 (1965).
6. W. H. Stone, R. G. Cragle, E. W. Swanson, D. G. Brown, *Science* **148**, 1355 (1965).
7. M. F. A. Woodruff and B. Lennox, *Lancet* **1959-II**, 476 (1959; J. O. W. Beilby, I. S. Cade, A. M. Jelliffe, D. W. Parkin, J. W. Stewart, *Brit. Med. J.* **1960-I**, 96 (1960).
8. D. B. Wilson, W. K. Silvers, P. C. Nowell, *J. Exp. Med.* **126**, 655 (1967).
9. R. E. Billingham, in *Transplantation of Tissues and Cells*, R. E. Billingham and W. K. Silvers, Eds. (Wistar Institute Press, Philadelphia, 1961), pp. 87–106.
10. D. B. Wilson, *J. Exp. Med.* **126**, 625 (1967).
11. R. E. Billingham, in *Transplantation of Tissues and Cells*, R. E. Billingham and W. K. Silvers, Eds. (Wistar Institute Press, Philadelphia, 1961), pp. 1–26.
12. R. E. Billingham and W. K. Silvers, *J. Cell Comp. Physiol.* **60** (Suppl. 1), 183 (1962).
13. W. K. Silvers, D. B. Wilson, J. Palm, *Science* **155**, 703 (1967).
14. E. A. Boyse, E. Stockert, L. J. Old, *Proc. Nat. Acad. Sci. U.S.* **58**, 954 (1967).
15. J. E. Murray, A. G. R. Sheil, R. Moseley, P. Knight, J. D. McGavic, G. J. Dammin, *Ann. Surg.* **160**, 449 (1964); E. White and W. H. Hildemann, *Science* **162**, 1293 (1968); R. Y. Calne, R. A. Sells, J. R. Pena, D. R. Davis, P. R. Millard, B. M. Herbertson, R. M. Binns, D. A. L. Davies, *Nature* **223**, 472 (1969).
16. Supported by PHS grant AI-07001. W.K.S. and D.B.W. are PHS career development awardees. D.M.L. is an NIH postdoctoral fellow. The technical assistance of J. Cavallaro is acknowledged.

13 November; revised 15 December 1969

## Oligodeoxyribonucleotides: Chemical Synthesis in Anhydrous Base

Abstract. *The sugar hydroxyl of a methoxytrityldeoxyribonucleoside reacts with a methoxytrityldeoxyribonucleoside phosphorofluoridate in dimethylformamide and potassium tertiary butoxide to yield the protected dinucleoside monophosphate. The reaction is fast and specific, and is used in a stepwise synthesis to prepare trinucleoside diphosphate and tetranucleoside triphosphate.*

Condensing agents such as dicyclohexylcarbodiimide (*1*), mesitylene sulfonyl chloride (*2*), and triisopropylbenzene sulfonyl chloride (*3*) are used usually for the synthesis of phosphodiesters in oligodeoxyribonucleotides. As a new approach to phosphodiester synthesis, Borden and Smith (*4*) described the synthesis of nucleoside-3′,5′ phosphates in strong anhydrous base. In this method the phosphate in the 3′ or 5′ position of a nucleotide bearing a suitable leaving group (*p*-nitrophenoxide) undergoes nucleophilic attack by the ionized 5′- or 3′- hydroxyl group of the sugar to form the intranucleotide bond. We now have used this principle to form the internucleotide bond. The reaction of suitably protected nucleosides and nucleotides is carried out with anhydrous potassium tertiary butoxide as the base and fluoride as the leaving group on the phosphate (Fig. 1). A stepwise synthesis is possible with the use of protecting groups of different stability in acid (Fig. 1, R and R′).

The preparation of TpT by this method was as follows. DMTr-$Tp_F$ (*6*) (20.4 μmole) and T-MMTr (10.7 μmole) in dimethylformamide (1 ml) were reacted with molar potassium tertiary butoxide in hexamethylphosphoramide (0.6 ml) for 15 minutes at room temperature. The reaction was stopped by addition of cation exchanger (Bio-Rad AG 50 W-X2, pyridinium form) (2 ml). The resin was filtered off and washed with 50 percent ethanol, and the combined filtrate was reduced to a gum by rotary evaporation. The methoxytrityl groups were removed by hydrolysis in 80 percent acetic acid (4 hours, 30°C) and subsequent ether extraction from a water solution. TpT was then isolated by DEAE-cellulose chromatography or by a combination of ion-exchange chromatography and paper chromatography (*7*). Based on the amount of T-MMTr used, the yield of TpT isolated was 90.5 percent.

Yields of TpT obtained under various reaction conditions are shown in Table 1. These indicate the following. (i) Dimethylformamide is a better solvent for this reaction than dimethylsulfoxide; (ii) the yields are relatively independent of the excess of potassium tertiary butoxide used in the reaction; (iii) high yields of TpT are obtained with only a twofold excess of the phosphorofluoridate over the nucleoside component; and (iv) the reaction is fast and probably complete in 10 minutes.

In these reactions, thymine was always one of the by-products, indicating breakdown of the glycosidic linkage. In a 15-minute reaction with a tenfold excess of base 2.3 percent of all glyco-

Table 1. Preparation of thymidylyl-(3′,5′)-thymidine. The starting compounds for these reactions were dissolved in 1 to 2 ml of the indicated solvent and reacted with potassium *t*-butoxide at room temperature. The products were isolated by ion-exchange chromatography and paper chromatography after the protecting groups had been removed by hydrolysis in acetic acid.

| T-MMTr reacted with | Nucleotide/ T-MMTr | Potassium *t*-buotoxide/ ionizing group | Solvent | Reaction time (min) | TpT formed (%) |
|---|---|---|---|---|---|
| DMTr-$Tp_F$ | 1.9 | 10 | DMF | 15 | 87 |
| DMT-$Tp_F$ | 2.1 | 10 | DMSO | 15 | 17 |
| MMTr-$Tp_F$ | 4.4 | 6 | DMF | 10 | 87 |
| DMTr-$Tp_F$ | 1.8 | 1.5 | DMF | 15 | 80 |
| DMTr-$Tp_F$ | 1.9 | 1.5 | DMF | 2 | 49 |