Europe. J. Cancer Vol. 15, 965–970.

0014-2964/79/0701-0965 $02.00/0

In Vitro and *In Vivo* effects of Rat Amniotic Fluid on Cell-Mediated and Humoral Immunity in Rats*

B. PARK, H. SOBIS, M. C. DELACOURT and M. VANDEPUTTE
Rega Institute for Medical Research, University of Leuven, 3000 Leuven, Belgium

Abstract—*Rat amniotic fluid has no immunosuppressive effect on T-lymphocyte stimulation by PHA and MLC as well as on B-lymphocyte stimulation by LPS.* In vivo *neither T-cell-mediated immunity against the oncogenic activity of polyoma virus nor T-B cell collaborative humoral immunity against sheep red blood cells in newborn rat was suppressed by rat amniotic fluid.*

Our experimental results indicate that rat amniotic fluid has no immunosuppressive effect on cell-mediated as well as on humoral immunity in the rat.

INTRODUCTION

THE FUNCTIONAL role of alpha-fetoprotein (AFP)-containing fluids and pure AFP has been a subject of active investigation. Several recent studies [1–4] suggest that AFP could be an important immunoregulatory factor in maternal–fetal relationship and may play a role in the protection of the fetus against maternal immune attack. This hypothesis is based on the results of experiments performed *in vitro* which show a definitive immunosuppressive effect of AFP-containing fluids and pure AFP on certain functions of thymus-derived (T) lymphocytes in mouse [1–6] and in human [7, 8]. However, several experimental results of other workers are not consistent with an immunosuppressive role of AFP-containing fluids and pure AFP [9–13]. Moreover, similar studies performed in the rat did not show an immunosuppressive effect of AFP on T-lymphocytes [11–13]. Because of the discrepancy in experimental findings we thought it worthwhile to investigate this subject *in vivo* as well as *in vitro*. Instead of using pure AFP, we preferred to study the effect of rat amniotic fluid (RAF) which is the fluid present in normal physiological condition during pregnancy.

Accepted 25 January 1979.
*Supported by the Belgian Algemene Spaar- en Lijfrentekas (ASLK) Cancer Fund and by the National Fonds voor Wetenschappelijk Onderzoek (NFWO).
†Correspondence to M. Vandeputte, Rega Institute for Medical Research, Minderbroeders-straat 10, B-3000 Leuven, Belgium.

In vitro we examined the effect of RAF on bone marrow-derived (B) lymphocyte function with lipopolysaccharide (LPS), and on T-lymphocyte functions with phytohemagglutinin (PHA) and mixed lymphocyte culture (MLC).

For the *in vivo* experiments, two different experimental models were used. In the first, the influence of RAF on T-B lymphocyte collaboration (T-lymphocyte-dependent antibody synthesis) was investigated by looking for the possibility to delay the neonatal period of unresponsiveness to sheep red blood cells (SRBC) using the plaque forming assay (PFC).

In the second model, we verified the effect of RAF on polyoma virus oncogenesis in the rat. Previous results have indeed shown that polyoma oncogenesis is very dependent on the T-cell-mediated immunity which develops with age [14–17].

MATERIALS AND METHODS

Animals

Rats of the inbred strains R (Wistar Albino) and BN (Brown Norway) as well as $(R \times BN)F_1$ hybrids were used.

RAF collection

Embryonic sacs of 14 days old pregnant R rats were punctured under sterile conditions. The amniotic fluids were collected, pooled,

centrifuged and stored at −70°C for further use.

Mitogen reactivity

Four × 10^5 spleen lymphocytes or lymph node cells (LNC) from R rats in 0.18 ml of MEM (Eagle's minimal essential media, Flow) supplemented with glutamine and 5% heat inactivated (56°C, 30 min) BN serum were cultured in microtitration plates (round-bottomed, 96 wells, Greiner) at 37°C in humidified atmosphere containing 5% CO_2 in air. To each well, at the beginning of cultivation, variable amounts of RAF or heat inactivated normal R rat serum (NRS) was added to obtain a 10, 1 and 0.5% final dilution. Twenty microgram/well of lipopolysaccharide-W (LPS, E. coli 055:B5, Difco, U.S.A.) or 2 μl/well of phytohemagglutinin (PHA, Wellcome reagent grade, 5 ml dried, Beckenham, U.K.) were added for lymphocyte stimulation. After 3 days of incubation, cells were labelled with 1 μCi/well of (^{3}H)TdR (Tritiated thymidine, SA; 20 Ci/mM, The Radiochemical centre, Amersham), and harvested 18 hr later with semi-automatic harvester (MASH II). The cell pellet on the filterpaper was dissolved at room temperature in 0.1 ml of Lumasolve (Lumac). After 4 hr 5 ml of Lipiluma (Lumac) were added. The radioactivity was counted by Packard Tri-Carb Liquid Scintillation spectrometer (Model 3375).

Mixed lymphocyte culture (MLC)

The MLC consisted of 2 × 10^5 R-LNC as responder cells and 4 × 10^5 (R × BN)F_1 − LNC as stimulator cells in 0.18 ml of MEM supplemented with glutamine and 5% heat inactivated BN serum. To each well, variable amounts of RAF or NRS were added at the beginning of cultivation to obtain a 10, 1 and 0.5% final dilution. After 5 days incubation, the cells were labelled with (^{3}H)TdR and harvested as described above.

Induction of polyoma virus tumors

R rats were inoculated subcutaneously with 5 × 10^6 plaque-forming units (PFU) polyoma virus per ml at the age of 2 days (group 1) or at 4 days (group 2). Half of the animals in each nest were injected intraperitoneally (i.p.) with RAF in increasing doses ranging from 0.1 to 0.4 ml each day between 2 and 12 days of age. The control litter mates were injected i.p. with the same amount of phosphate buffered saline (PBS).

In vivo *primary antibody response (PFC) to SRBC (sheep red blood cell)*

Assays were made by the method of Jerne hemolytic plaque assay [18, 19]. As our experimental data showed that *in vivo* primary antibody response (IgM) to SRBC started at day 10 or 12 after birth in R rats (unpublished data), we immunized R rats which were 12 days old with 4 × 10^8 SRBC i.p. The rats were injected i.p. with RAF or with PBS (control litter mates) from day 2 till day 16 after birth in increasing doses from 0.1 to 0.5 ml daily. Five days after immunization with SRBC, the PFC assays were performed.

RESULTS

Mitogen responses

The influence of different amounts of RAF and NRS on the response of spleen cells to LPS is shown in Table 1. From these data, it is evident that RAF, firstly, increases the

Table 1. Effect of RAF on response of R rat spleen cell culture to LPS

Preparation	(^{3}H) TdR incorporation	
	No stimulant	LPS
	mean counts/min ± S.D.*	
Control (media)	1191.0 ± 240.6	9233.0 ± 1837.5
RAF (10%)†	5475.9 ± 920.2	34,939.0 ± 1255.0
RAF (1%)	2711.4 ± 107.2	10,716.0 ± 522.0
RAF (0.5%)	2436.7 ± 423.9	7657.3 ± 566.4
NRS (10%)	684.5 ± 89.0	7716.0 ± 1437.7
NRS (1%)	1213.4 ± 180.0	6376.5 ± 2073.7
NRS (0.5%)	1279.7 ± 837.5	6164.2 ± 233.0

*Mean counts/min; mean of 4 wells. S.D.; standard deviation
†Final dilutions in cultures.

thymidine incorporation in the unstimulated as well as in LPS stimulated cultures; secondly increases rather than inhibits the reactivity of LPS. In the PHA stimulation test with lymph node cells (Table 2), RAF has a slight mitogenic effect on unstimulated culture but no definitive effect on the stimulated culture. The effect of RAF on MLC is shown in Table 3. In this test again no suppressive effect of RAF was observed. Instead a rather stimulating effect at least with high amounts of RAF was recorded.

Effect of RAF on the development of polyoma virus-induced tumors

The results presented in Table 4 show no significant difference in the incidence of tumors between control and experimental rats of both groups (groups 1 and 2). It may be concluded from these data that RAF has no immunosuppressive effect on the T-cell-mediated immunity against the oncogenic activity of polyoma virus in rats.

Effect of RAF on in vivo *primary antibody response (PFC) to SRBC*

As shown in Table 5, there is no significant difference between the number of PFC of 12 days old R rats which were injected with RAF and those of control.

DISCUSSION

Alpha-fetoprotein is a serum protein which is found in high concentration in fetal sera, maternal sera during pregnancy, in the sera of hepatoma and yolk sac carcinoma-bearing animals, and in amniotic fluid [22–26]. It is synthesized by the fetal liver and yolk sac and

Table 2. *Effect of RAF on response of R rat LNC culture to PHA*

Preparation	(^{3}H) TdR incorporation	
	No stimulant	PHA
	mean counts/min ± S.D.*	
Control (media)	176.9 ± 41.2	52,547.7 ± 4621.0
RAF (10%)†	619.6 ± 56.8	55,979.6 ± 8033.2
RAF (1%)	233.3 ± 25.3	38,212.0 ± 3019.6
RAF (0.5%)	225.9 ± 10.1	37.093.6 ± 4467.0
NRS (10%)	91.5 ± 17.6	62,327.0 ± 3735.1
NRS (1%)	80.9 ± 2.6	42,528.0 ± 4429.4
NRS (0.5%)	70.7 ± 19.7	36,054.5 ± 4868.0

*Mean counts/min; mean of 4 wells. S.D.; standard deviation
†Final dilutions in culture.

Table 3. *Effect of RAF on MLC of R− and F_1 (R+BN) LNC*

Preparation	(^{3}H) TdR incorporation	
	Responding cell only (R − LNC)	R − LNC + F_1 − LNC
	mean counts/min ± S.D.*	
Control (media)	265.2 ± 8.7	14,907.5 ± 2655.2
RAF (10%)†	747.3 ± 82.3	49,860.4 ± 5710.7
RAF (1%)	92.2 ± 21.2	16,514.8 ± 4492.5
RAF (0.5%)	208.8 ± 27.9	19,670.4 ± 1321.9
NRS (10%)	960.3 ± 70.0	16,278.4 ± 1177.6
NRS (1%)	61.3 ± 8.6	15,877.2 ± 4343.9
NRS (0.5%)	100.7 ± 9.0	13,262.0 ± 1573.0

*Mean counts/min; mean of 4 wells. S.D.; standard deviation.
†Final dilution in culture.

Table 4. Effect of RAF on induction of polyoma virus tumors in R rats

Age at virus inoculation* (days)	Treatment	Number inoculated	Number of rats with tumors
2	PBS	10	7
(group 1)	RAF	10	9
4	PBS	11	5
(group 2)	RAF	11	1

*Each rat received s.c. 0.1 ml of polyomavirus (5×10^6 PFU/ml).

Table 5. Effect of RAF on in vivo *antibody response (PFC)* to SRBC in R rats*

Experiment	Mean No. PFC/2.5×10^6 spleen cells ± SD† PBS	RAF
1	23.0 ± 2.6	19.5 ± 4.5
2	26.5 ± 4.5	29.7 ± 5.0
3	50.7 ± 4.2	47.0 ± 9.2

*PFC; plaque forming cell
†Mean value of 4 petri dishes ± standard deviation.

secreted into the fetal and maternal circulation [25].

The physiological role of AFP as well as of the other components present in amniotic fluid, however, remains obscure. Although an immunosuppressive effect of AFP and amniotic fluid has been reported in the mouse, no such positive results were found in the rat [11–13].

Nearly all these data were obtained from experiments performed *in vitro*. If amniotic fluid has an immunosuppressive function in normal physiological conditions it is likely to show this effect also *in vivo*. Therefore we studied the influence of amniotic fluid both *in vivo* and *in vitro*. As reported in the results, we could not detect an immunosuppressive effect either *in vitro* or *in vivo*. One may speculate that if AFP is really the immunosuppressive factor in amniotic fluid, the amount used in our experiments was too small. This is not likely since as reported by Colquhoun *et al.* [27], the AFP concentration in amniotic fluid at day 14 of gestation is 743 μg/ml. Considering the dilutions of RAF used in the *in vitro* experiment, this represents an AFP concentration in the range of 3.7–74 μg/ml. These concentrations of AFP correspond to the ones used by other authors [2].

By comparing the influence of these different dilutions of RAF to those of NRS, it is clear that there is no immunosuppressive effect of RAF in *in vitro* B-lymphocyte stimulation by LPS as well as T-cell stimulation by PHA or semiallogeneic stimulator cells (MLC).

Recent studies by Sell *et al.* [11, 28] in the rat also showed no difference between normal autologous serum and AFP-rich sera on the T-cell stimulation by Con. A, PHA or allogeneic stimulator cells.

The failure to show the immunosuppressive effect of rat AFP has also been reported by Parmely *et al.* [12] and Belanger *et al.* [13] who stressed that the immunosuppression of purified rat AFP may be caused by an artifact related to protein isolation procedures or culture conditions.

All these observations including the results of this report do not show an immunosuppressive effect of AFP-containing fluids or pure AFP *in vitro* in the rat. However, recent studies of Murgita *et al.* [1, 2] in the mouse show quite contradictory observations. Their findings indicate a uniformly and dose-dependent immunosuppressive effect of mouse amniotic fluid and pure AFP on LPS, PHA and MLC reactions of mouse spleen cells. These observations are also supported by the reports made by Keller *et al.* [6] and Zimmerman *et al.* [5].

Regarding these contradictory findings between mouse and rat, it is quite possible that species differences may be responsible for these

different results on *in vitro* experiments. Whatever the laboratory animals used, however, the physiological role of AFP-containing fluids should also be verified on the basis of *in vivo* obtained experimental results.

To investigate the immunosuppressive effect of RAF on *in vivo* T-cell-mediated immune response, we verified its effect on the oncogenic activity of polyoma virus in the rat. As mentioned previously, the development of an effective T-cell-mediated immunity against the oncogenic activity of polyoma virus gradually appears during the first week of life in the rat. Since even a slight interference with the development of the T-cell-mediated immunity markedly increases the tumor incidence, we used this model to investigate a possibile inhibition of the cell-mediated immunity by RAF. As shown in Table 4, the injection of gradually increasing doses of RAF did not increase the tumor incidence. This negative result is not likely to be due to an insufficient amount of AFP injected. Indeed, as reported by Colquhoun *et al.* [27], the half-life of AFP in newborn rats is approximately 5 days until day 26 after birth. Therefore we can be confident that the rats treated with RAF from day 2 until day 12 of life received quite a high amount of RAF.

The same remarks apply to the experiment in which the influence of amniotic fluid was verified on the *in vivo* T–B cell collaboration in the production of plaque forming cells against sheep red blood cells. As the immunological unresponsiveness to SRBC is terminated on the 11th (± 1) day of age in R rats, we looked for any possible prolongation of this immunological unresponsiveness to SRBC by the repeated injection of RAF with increasing doses from day 2 until day 16 after birth. We failed to find any prolongation of this period of unresponsiveness to SRBC (Table 5). The effect of AFP-rich sera on antibody formation *in vivo* to SRBC has also been reported by Sell *et al.* [11]. These authors could not find a difference in the sheep cell agglutinin response between rats injected with normal rat serum and rats treated with sera of hepatoma-bearing animals rich in AFP. All these findings indicate that RAF or sera rich in AFP do not influence the *in vivo* humoral immune response.

Recent studies of Umiel *et al.* [20] and Ptak *et al.* [21] suggest the importance of generation of suppressor cells in neonatal immunological hyporeactivity. If AFP is operative in the induction of suppressor T-cells [3], one would expect a prolongation of neonatal hyporeactivity by RAF. However, from our *in vivo* experiments, neither suppression of T-cell-mediated immunity against the polyoma virus nor prolongation of the immunological unresponsiveness to SRBC was observed.

In conclusion, we could not detect an immunosuppressive effect of RAF using different *in vitro* and *in vivo* models in the rat.

Whether RAF and AFP play an important role as an immunoregulatory factor during pregnancy is until now not proven. Most of the available experimental data indicate that, at least in the rat, this immunosuppressive function of AFP and RAF is not observed.

Acknowledgements—We are indebted to Mr. C. Seghers for skillful technical assistance. The editorial assistance of Miss C. Callebaut is appreciated.

REFERENCES

1. R. A. Murgita and T. B. Tomasi, Jr., Suppression of immune response by alpha-fetoprotein. I. The effect of mouse alpha-fetoprotein on the primary and secondary antibody response. *J. exp. Med.* **141,** 269 (1975).
2. R. A. Murgita and T. B. Tomasi, Jr., Suppression of immune response by alpha-fetoprotein. II. The effect of mouse alpha-fetoprotein on mixed lymphocyte reactivity and mitogen-induced lymphocyte transformation. *J. exp. Med.* **141,** 440 (1975).
3. R. A. Murgita, E. A. Goidl, S. Kontiainen and H. Wigzell, Alpha-fetoprotein induces suppressor T-cell *in vitro*. *Nature (Lond.)* **267,** 257 (1977).
4. A. B. Peck, R. A. Murgita and H. Wigzell. Cellular and genetic restrictions in the immunoregulatory activity of alpha-fetoprotein. I. Selective inhibition of anti-IA-associated proliferative reactions. *J. exp. Med.* **47,** 667 (1978).
5. E. Zimmerman, M. Voorting-Hawking and J. G. Michael, Immunosuppression by mouse sialylated alpha-fetoprotein. *Nature (Lond.)* **265,** 354 (1977).
6. R. H. Keller and T. B. Tomasi, Jr., Alpha-fetoprotein synthesis by murine lymphoid cells in allogeneic reactions. *J. exp. Med.* **143,** 1140 (1976).

7. S. Yachnin, Demonstration of the inhibitory effect of human alpha-fetoprotein on *in vitro* transformation of human lymphocytes. *Proc. nat. Acad. Sci. (Wash.)* **73,** 2857, (1976).
8. S. Gupta and R. A. Good, Alpha-fetoprotein and human lymphocyte subpopulation. *J. Immunol.* **18,** 405 (1977).
9. B. H. Littman and E. Alpert, The effect of purified alpha-fetoprotein on *in vitro* assays of cell-mediated immunity. *Cell. Immunol.* **30,** 35 (1977).
10. H. W. Sheppard, Jr., S. Sell, P. Trefts and P. Bahu, Effects of alpha-fetoprotein on murine immune responses. I. Studies on mice. *J. Immunol.* **119,** 91 (1977).
11. S. Sell, H. W. Sheppard, Jr. and M. Poller, Effects of α-fetoprotein on murine immune responses. II. Studies on rats. *J. Immunol.* **119,** 98 (1977).
12. M. J. Parmely and J. S. Thompson, Effects of α-fetoprotein and other serum factors derived from hepatoma bearing rats on the mixed lymphocyte response. *J. Immunol.* **117,** 1832 (1976).
13. L. Belanger, F. Daguillard, W. I. Waithe and D. Dufour, Studies on the effect of AFP on immune status of children with tyrosinosis and on mitogen induced rat and human lymphocyte transformation *in vitro*. In *Oncofetal Gene Expression.* (Edited by W. H. Fishman and S. Sell) p. 329. Academic Press, New York (1976).
14. M. Vandeputte and P. De Somer, Oncogenic activity of the polyoma virus in thymectomized rats. *Life Sci.* **2,** 475 (1963).
15. M. Vandeputte, H. Eyssen, H. Sobis and P. De Somer, Induction of polyoma virus tumors in athymic nude mice. *Int. J. Cancer* **14,** 445 (1974).
16. M. Vandeputte and P. De Somer, Influence of thymectomy on viral oncogenesis in rats. *Nature (Lond.)* **206,** 520 (1965).
17. M. Vandeputte, Antilymphocytic serum and polyoma oncogenesis in rats. *Transplant. Proc.* **1,** 100 (1969).
18. N. K. Jerne, A. A. Nordin and C. Henry, The agar plaque technique for recognizing antibody producing cells. In *Cell-Bound Antibody.* (Edited by Amos and Koprowski) p. 109. Wistar Institute Press, Philadelphia (1963).
19. L. Hudson and F. C. Hay, *Practical Immunology*, p. 57. Blackwell Scientific Publication, Oxford, U.K. (1976).
20. T. Umiel, A. Globerson and N. Trainin, Development of immunosuppressor cells. *Transplantation* **24,** 282 (1977).
21. W. Ptak and A. Skowron-Cendrzak, Fetal suppressor cells. *Transplantation* **24,** 45 (1977).
22. G. I. Abelev, Alpha-fetoprotein in oncogenesis and its association with malignant tumors. *Advanc. Cancer Res.* **14,** 295 (1971).
23. S. Sell, F. F. Becker, H. L. Leffert and H. Watabe, Expression of oncodevelopmental gene product (alpha-fetoprotein) during fetal development and adult oncogenesis. *Cancer Res.* **36,** 4239 (1976).
24. R. Hooghe, M. Zeicher, H. Sobis and M. Vandeputte, Yolk sac carcinomas induced by murine sarcoma virus and producing alpha-fetoprotein. *Proceedings of the International Conference for Alpha-Fetoprotein.* (Edited by R. Masseyeff) p. 271 (1974).
25. D. Gitlin and M. Boesman, Fetus specific serum proteins in several mammals and their relationship to human alpha-fetoprotein. *Comp. Biochem. Physiol.* **21,** 327 (1967).
26. S. Sakashita, Y. Tsukada, K. Nakamura, I. Tsuji and H. Hirai, Experimental yolk-sac tumors produced by fetectomy without virus infection in rats. *Int. J. Cancer* **20,** 83 (1977).
27. B. Colquhoun, P. Gold, W. Barzell, D. D. Oakes and J. Shuster, Metabolism of rat α_1-fetoprotein. *Cancer Res.* **34,** 2296 (1974).
28. H. W. Sheppard Jr., S. M. Poler, P. Trefts and S. Sell, Effect of AFP on immune phenomena in rats and mice. In *Oncofetal Gene Expression.* (Edited by W. H. Fishman and S. Sell) p. 317. Academic Press, New York (1976).