cated areas show numerous blood vessels (figure 4). During further development these lobules differentiate into adipose tissue.

Despite the complete absence of musculature in the distal parts of the limbs, tendons have differentiated. Their distal ending attaches to the perichondrium of the phalanges, while the proximal part passes progressively into the loose mesenchyme of the muscle-forming zone (figure 5). These observations are in conformity with previous findings on the autonomy of tendon development[12-14].

The results of the 2 experimental series show that the avian somatopleural mesoderm gives rise to skeletal elements, smooth muscles, tendons and connective tissues. However, skeletal muscle fibres do not differentiate from somatopleural cells. The fact that hybrid myotubes cannot be found within the operated regions supports our point of view that the myoblastic component of the muscles solely originates from the somites. Since the somatopleural fragments after heterotopical cultivation exhibit no musculature, it may be excluded that the somatopleural cells undergo a change in their programme of development in order to compensate for somitic deficiency. This is in line with the findings of Dienstman et al.[15] which indicate that the mesoderm cells of the early limb bud are already determined as cartilage or muscle precursor cells.

Our results are in partial contradiction to those reported by Chevallier et al.[9,10]. After removal of the brachial somitic mesoderm these authors observed muscles within the wing. Moreover, after the replacement of quail somites by chick somites they found muscle bulks of mixed constitution. In our opinion these observations offer no proof that the somatopleure gives rise to muscle fibres, because it was expressly conceded by the authors that the somites of the host embryos have not been completely removed. With regard to the remarkable capacity of the somitic mesoderm to regulate deficiencies[16], it is worth considering that from the remaining somitic parts the migration of myogenic cells can still occur.

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## Correlation of immunogenicity with suppression of lymphocyte adenosine 3′,5′-monophosphate-dependent protein kinase

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*Summary.* Cyclic-AMP-dependent protein kinase activity was depressed in whole spleen as well as in isolated splenic lymphocytes from 3-methylcholanthrene (MCA), R3230 AdCa mammary adenocarcinoma, N-hydroxy-2-acetylaminofluorene, and 4-dimethylaminoazobenzene (DMAAB) tumor-bearing Fischer rats as compared to control animals. The magnitude of depression increased with the immunogenicity of the tumor. The depressed enzyme activity was the result of a reduced $V_{max}$ for adenosine 3′,5′-monophosphate (cAMP)-stimulated histone phosphorylation.

Adenosine 3′,5′-monophosphate (cAMP) plays an important role in regulation of the immune responsiveness[2,3]. There is extensive evidence to indicate that cAMP inhibits immune responsiveness which leads to enhanced growth[4-7]. cAMP mediates its physiological effects by cAMP-dependent protein kinase[8]. The present studies report on cAMP-dependent protein kinase in whole spleens and spleen lymphocytes of normal and tumor-bearing rats. The tumors examined possessed various degree of antigenicity so that the cAMP-dependent protein kinase and immunogenicity of the neoplasm could be correlated.

*Materials and methods.* Tumors. 4 tumor systems, sarcoma MCA, mammary R3230 adenocarcinoma (R3230 AdCa), mammary carcinoma AAF and hepatoma DMAAB (table 1) were induced and carried on in inbred Fischer rats (80–90 g) which initially were obtained from Charles River Breeding Laboratory (Wilmington, Mass.).

Rat splenic lymphocytes were obtained from a Ficoll-Hypaque gradient (8 ml of 8% Ficoll plus 2 ml of 50% Hypaque) as described by Simon et al.[9]. The cells were next washed 2 times in hypotonic Tris buffer (2.00 g Tris plus 7.47 g ammonium chloride/l, pH 7.2) to remove contaminating erythrocytes[10]. The final cell preparations had more than 98% small lymphocytes (as indicated by Wright's Stain) and 95% were viable (as determined by trypan blue dye exclusion).

Preliminary experiments showed that the supernatant fraction resulting from a 50,000 × g centrifugation (20 min) of spleen lymphocytes homogenized in 20 mM 2 N-morpholineethanol sulfonic acid (MES) buffer, pH 7.0, contained over 85% of the cAMP-dependent protein kinase in whole homogenate. In brief, the protein kinase reaction[11] was initiated by addition of the enzyme preparation to a reaction mixture containing: 0.5 mM EGTA, 10 µg calf thymus type II-A histone, 2.0 mM theophylline, 5.0 mM sodium fluoride, 0.01% bovine serum albumin (BSA), 30.0 mM $MgCl_2$, 20.0 mM MES, 0.1 mM ATP containing $5 \times 10^6$ cpm of gamma labeled $^{32}$P-ATP, and with or

Table 1. Properties of the tumors in unconditioned Fischer rats

| Tumor | Method of induction Agent | Mode and cell number* | Immunogenicity Magnitude | Tumor cells rejected | Responsiveness to hormones |
|---|---|---|---|---|---|
| Sarcoma-MCA | 3-Methylcholanthracene | s.c. $5\times10^4$ | High | $1\times10^7$ | None |
| R3230 AdCa | Spontaneous | s.c. $3\times10^3$ | Undetectable | $<1\times10^3$ | Responsive independent** |
| AAF | N-hydroxy-2-acetylaminofluorene | i.p. $1\times10^3$ | Undetectable | $<1\times10^3$ | Responsive dependent |
| DMAAB | 4-dimethylaminoazobenzene | Oral $5\times10^3$ | Moderate | $1\times10^4$ | None |

* Number of cells needed to produce tumor. ** Tumor growth in vivo, or tumor cell proliferation in vitro is enhanced by estradiol, but it does not depend on the presence of the estrogen. The tumor grows in vivo and tumor cells proliferate without injection of estradiol or its presence in the culture medium, respectively.

Table 2. Adenosine 3′,5′-monophosphat in tumor cells

| Tumor | Immunogenicity* | Cyclic nucleotides (pmoles of the nucleotide/μg DNA) cAMP | cGMP | Adenylate cyclase** |
|---|---|---|---|---|
| Sarcoma-MCA | $5\times10^8$ (0/15) | 78.7 ± 4.9 | 48.8 ± 3.1 | 35.9 ± 2.6 |
| R3230 AdCa | $1\times10^3$ (2/15) | 28.5 ± 1.7 | 14.6 ± 0.8 | 10.8 ± 1.3 |
| Mammary carcinoma (AAF) | $1\times10^3$ (15/15) | 19.6 ± 0.9 | 10.9 ± 0.5 | 6.7 ± 0.3 |
| Hepatoma-DMAAB | $5\times10^3$ (0/15) | 57.6 ± 3.5 | 37.6 ± 2.2 | 17.8 ± 1.9 |

* Immunogenicity is expressed as the number of cells per challenging dose which the animals rejected. Animals which developed tumor: total number of animals challenged is presented in parenthesis. ** Adenylate cyclase is expressed as pmoles cAMP per mg protein per min. The data is presented as the mean ± SD of 6 separate assays.

Table 3. Protein kinase and immunogenicity of tumor cells

| | Protein kinase activity (pmoles $^{32}$p-incorporated/mg protein/minute) Endogeneous proteins With cAMP | Without cAMP | Histone With cAMP | Without cAMP | Casein With cAMP | Without cAMP |
|---|---|---|---|---|---|---|
| Sarcoma-MCA | 463 ± 38 | 174 ± 22 | 2976 ± 208 | 1476 ± 147 | 2143 ± 168 | 1743 ± 121 |
| R3230 AdCa | 85 ± 9 | 35 ± 5 | 747 ± 87 | 349 ± 46 | 543 ± 46 | 462 ± 43 |
| Mammary carcinoma (AAF) | 63 ± 6 | 27 ± 4 | 478 ± 67 | 187 ± 31 | 417 ± 57 | 338 ± 29 |
| Hepatoma-DMAAB | 145 ± 12 | 58 ± 7 | 1368 ± 114 | 693 ± 73 | 1037 ± 93 | 875 ± 67 |

The results represent the mean ± SD of 4 separate assays.

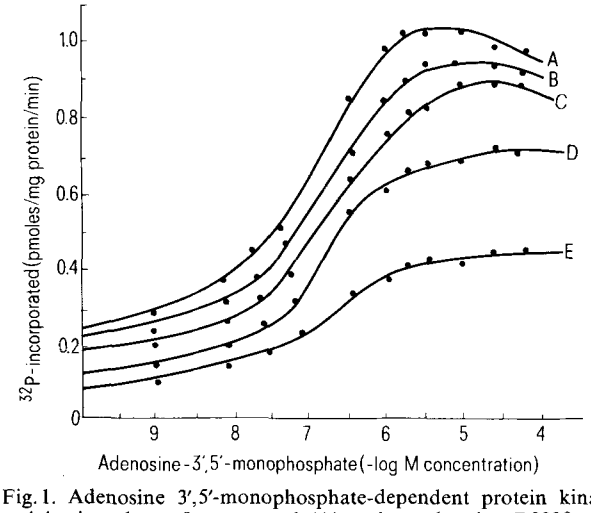


Fig. 1. Adenosine 3′,5′-monophosphate-dependent protein kinase activity in spleens from control (A) and rats bearing R3230 AdCa (B), AAF (C), DMAAB (D) and MCA (E). (p-values less than 0.05, 0.01, 0.005 and 0.001 for R3230 AdCa, AAF, DMAAB and MCA.)

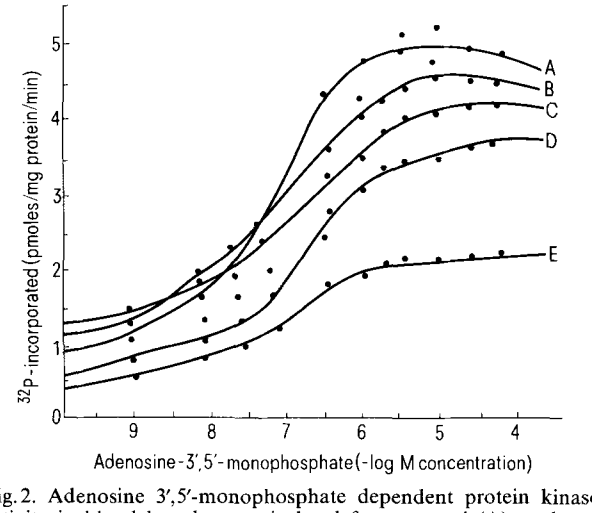


Fig. 2. Adenosine 3′,5′-monophosphate dependent protein kinase activity in blood lymphocytes isolated from control (A), and rats bearing R3230 AdCa (B), AAF (C), DMAAB (D) and MCA (E) tumors. (p-values less than 0.1, 0.05, 0.005 and 0.01 for lymphocytes from R3230 AdCa, AAF, DMAAB and MCA-bearing animals.)

without added cAMP. The TCA precipitable $^{32}P$ was counted on a Packard liquid scintillation spectrometer and the total pmole of phosphate incorporated from the known specific activity of $^{32}P$-ATP in the reaction mixture was calculated. Fischer rats carrying sarcoma MCA, R3230 AdCa, mammary carcinoma AAF and DMAAB hepatoma for 27 days (tumor weight was 2–5 g) and control rats were bled then sacrificed by cervical dislocation and their spleens removed into cold HBSS. Spleen cells and spleen lymphocyte suspensions were prepared and homogenized in MES buffer. Aliquots of the $50{,}000 \times g$ supernatants were assayed for the cAMP-dependent protein kinase activity at varying concentrations of cAMP. Statistical significance of the data was determined by Paired Student's test using $n=4$.

*Results and discussion.* The protein kinase assay was standardized using the enzyme from normal rat spleen cells and spleen lymphocytes. The reaction was linear over the range of 1.25 to $20 \times 10^5$ cells, has a $K_m$ for histone phosphorylation of 10 µg/ml and has $K_a$ and $V_{max}$ values for cAMP activation of 3 mM and 17.8 pmoles/min/$1 \times 10^6$ cells, respectively. The pH optimum is 7.0 and the reaction is linear from 25 to 40 °C.

The results in figure 1 show that there is a loss of cAMP-dependent protein kinase activity in spleens of tumor-bearing animals. The decrease was highest in CMA-bearing (highly immunogenic) and lowest in R3230 AdCa-bearing rats (lack detectable immunogenicity). The results in figure 2 show that blood lymphocytes from tumor-bearing animals displayed a decrease in cAMP-dependent protein kinase activity. The magnitude of the decrease was highest in lymphocytes of CMA-bearing and lowest in lymphocytes of R3230 AdCa-bearing rats. There appears to be a correlation between the loss of cAMP-dependent protein kinase activity and the immunogenicity of the tumor.

Studies on enzyme variations as a consequence of cell-mediated immune response should yield useful information regarding this complex phenomenon. Studies from these laboratories[4,7,8] demonstrated that when incorporated into the culture media of human mammary adenocarcinomas, protease inhibitors accelerated cAMP accumulation and sustained proliferation of human neoplastic cells. The results obtained from the described experiments indicate that cAMP-dependent protein kinase is depressed in whole spleens and lymphocytes of tumor-bearing animals. The magnitude of depression increased with the immunogenicity of the tumor. The results in table 2 indicate a possible relationship between accumulation of cAMP in and immunogenicity of the tumor cells. Although the level of cAMP is lowered in transformed cells[13–15], it is definitely increased in progressively growing tumors. The magnitude of the increase is inversely proportional to the immunogenicity of the tumor.

The results in table 3 summarize the relationship between protein kinase activities of the tumor cells on various substrates in presence and absence of cAMP. All the 4 tumors contained cAMP-dependent protein kinase. The enzyme activities were highest in phosphorylating histone. The magnitude of the activity varied with the immunogenicity of the tumor. Studies of the enzyme specific activities of adenosine 3′,5′-monophosphate-dependent protein kinase in tumor cells and spleen lymphocytes of tumor-bearing animals are in progress.


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## Cell division and cell death during regression of the chick embryo Müllerian ducts


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*Summary.* In 9-day-old chick embryos, decreased DNA synthesis and enhanced necrosis were observed in a defined area of the right female Müllerian ducts, supporting the idea of the existence of a regression process in this organ. In the male ducts, decreased DNA synthesis and a low level of necrosis were present all over the studied portion of the organ.


The mechanism of the regression of the Müllerian duct (MD) is not well understood and there are opposite views concerning the regression of the right female duct. Groenendijk-Huijbers[3] has called it the 'female-type regression', others[4,5] however, claimed that the organ does not regress but merely stops growing. Necrosis in the right female duct has been previously reported[6]. In the present work DNA synthesis is measured in different areas of the MD to see whether the rate of cell division is affected all over the organ, or if it is localized in preferential zones.

*Materials and methods.* MD from 9-day-old chick embryos were excised still attached to the mesonephros. In this way it was possible to manipulate the anterior region of the MD without touching it directly. Because of handling difficulties, the posterior part of the organ was not studied. For comparison with the MD of 9-day-old embryos, ducts of 8-day-old and 10-day-old embryos were also analyzed. The genetically sexed[7] 8-day-old embryos were kindly supplied by Dr J.M. Gasc (Institut d'Embryologie du C.N.R.S. et du Collège de France, Nogent-sur-Marne, France). The incorporation of tritiated thymidine (40 µCi $^3$H-methyl thymidine/ml tyrode solution, pH 7.2, s.a. 25 Ci/mmole, CEA Saclay, France) was carried out for 1 h in an incubator at 38 °C with constant shaking. The MD were fixed in 95%