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Original Article

Caudalization by retinoic acid is correlated with inhibition of cell population growth and expansion of chick blastoderms cultured *in vitro*

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ABSTRACT **Full primitive streak stage chick embryos, cultured *in vitro* for 20 h in the presence of 10^{-9} to 10^{-7} moles of retinoic acid (retinol, all-trans), exhibit increasing extent of malformations. RA causes caudalization, suppression of telencephalon, formation of open and enlarged neural tube in the regions of midbrain, hindbrain and spinal cord, failure of fusion of heart tubes, and gives rise to winged or diffused, and even supernumerary somites. Extreme abnormalities include failure to form the head fold and foregut. Abnormal embryos were graded according to Rao and Chauhan (*Teratology 4:* 191-198, 1971), and we find that the larger the severity of malformation, the smaller the size of total cell population and blastoderm area. Concomitant to caudalization, retinoic acid suppresses the cell population growth.**

KEY WORDS: *retinoic acid, chick embryo, caudalization, cell population growth inhibition, teratogenesis*

Introduction

High doses of vitamin A or its derivatives induce abnormal development (Cohlan, 1953; Kalter and Warkany, 1959; Kochhar, 1967). Retinoic acid (RA) causes retinoic acid embryopathy (Lammer *et al.*, 1985) exhibiting limb abnormalities, defects in the heart and thymus, cleft palate and other craniofacial defects. Acting through specific receptors (Giguere *et al.*, 1987, 1989; Petkovich *et al.*, 1987; Zelent *et al.*, 1989; Ellinger-Ziegelbauer and Dreyer, 1991) and cellular binding proteins (Maden *et al.*, 1989; Perez-Castro *et al.*, 1989), RA can alter the expression of specific genes (LaRosa and Gudas, 1988), or induce the expression of different homeobox genes (Dolle *et al.*, 1989). The recent view is that RA is a morphogen, or a primary morphogenetic determinant, acting as a posteriorizing/caudalizing substance (Durston *et al.*, 1989; Eichele, 1989; Green, 1990) and, similar to LiCl, it alters the expression of pattern forming or homeobox genes (Scott *et al.*, 1989; Simeone *et al.*, 1990, 1991; Sundin and Eichele, 1992) leading to abnormal morphogenesis and reduced embryonic size. We have earlier shown that the cell population growth is exponential during the early morphogenesis of chick embryos *in ovo* (McMaster and Modak, 1977) as well as *in vitro* (Ghatpande *et al.*, 1990). Our recent studies (Ghatpande *et al.*, 1991, 1993) show that the severity of abnormal development induced by LiCl or trypan blue is correlated with suppression of cell population growth and blastoderm expansion in chick embryos. In this paper, we show that RA suppresses cell population growth and area expansion concomitant to abnormal morphogenesis in chick embryo.

Results

With 10^{-7} moles of RA, all treated embryos were abnormal (Figs. 1 and 2), while with doses decreasing from 10^{-8} to 10^{-9} moles, the frequency of malformed embryos decreased to 80% and 11.5%, respectively (Fig. 3). RA-treated abnormal embryos exhibiting some of the morphological features present in normal embryos were identified as closest to the nearest equivalent normal Hamburger and Hamilton (1951) stage and their frequency distribution is shown in Fig. 3, with the insets showing the distribution according to the grade of abnormality. The observed abnormalities include (a) complete absence of brain morphogenesis and absence of head-fold formation (Fig. 1a,b), (b) reduced or open forebrain (Figs. 1c,e,f and 2a,b) and absence of telencephalon (Fig. 1g), (c) open and enhanced midbrain (Fig. 2a,b) and hindbrain regions (Figs. 1d and 2a,b) and open neural tube (Figs. 1c-e and 2b), (d) abnormal heart development, often with partially fused cardiac tubes (Figs. 1c,e and 2d) along with a widened foregut which may even be absent. In most cases, the somite primordia are positioned bilaterally (Figs. 1a,b and 2a), sometimes with winged (Figs. 1c,f,g and 2b), diffuse (Figs. 1e, 2b) or even an extra somite (Fig. 2d). Examples of embryos with grade I malformations are shown in Fig. 1a,b. Grade II embryos are found with (Figs. 1d and 2b) or without (Figs. 1c and 2a) head-

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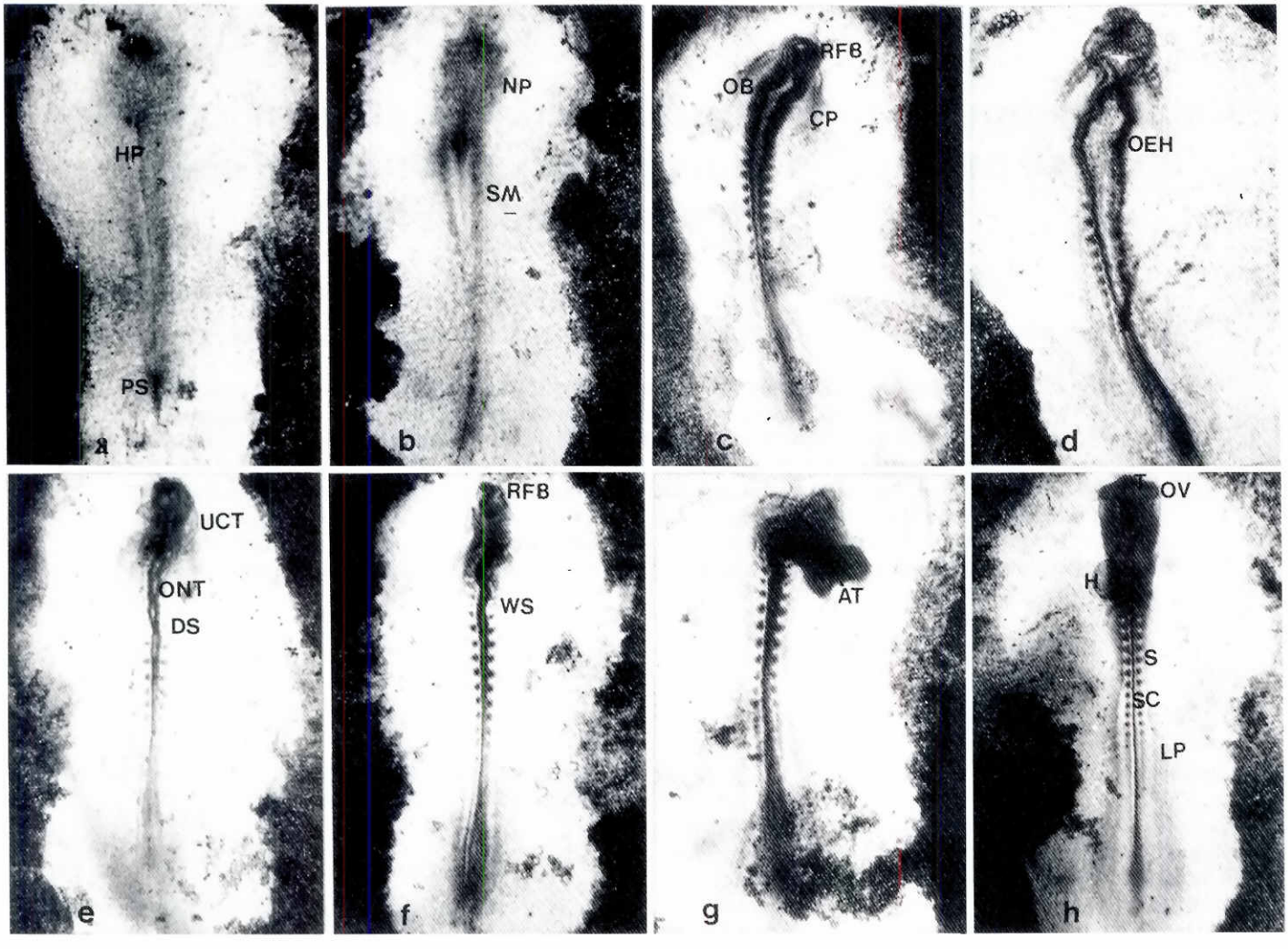

Fig. 1. Effect of 10^{-7} moles of retinoic acid on the development of primitive streak stage chick embryo treated *in vitro*. for 20 h. (a,b) *Embryos with 7 rank value and showing grade I abnormalities with head process (HP), neural plate (NP) with anterior thickening, bilaterally symmetrical somite mesoblast (SM) and regressed primitive streak (PS).* **(c)** *(Rank= 20) and* **(d)** *(rank= 21) show grade II abnormalities with open brain (OB), lack of head-fold formation and reduced forebrain area (RFB), open and enlarged hind brain (OEH), unfused cardiac primordia (CP) and well formed paired somites.* **(e)** *(Rank= 22) and* **(f)** *(rank= 29) are grade III embryos with reduced forebrain (RFB), open neural tube (ONT), diffuse (DS) or winged (WS) somites and unfused cardiac tubes (UCT).* **(g)** *Grade IV embryo (rank= 33) showing absence of telencephalon (AT).* **(h)** *Control normal embryo (rank= 37) of stage 11 with forebrain (FB), telencephalon (T), optic vesicles (OV), mid brain, hind brain, spinal cord (SC), foregut, heart (H), 12 pairs of somites (S) and lateral plate mesoderm (LP). (r= rank value).*

fold formation but with differentiated neural tissue. Embryos of grade III abnormalities are seen in Figs. 1e,f and 2c,d, while Fig. 1g represents a grade IV embryo. For each embryo the estimated rank value *(r)* is also shown in Figs. 1 and 2.

A comparison of Hamburger and Hamilton stage-wise distributions of controls, and RA-treated embryos show (Fig. 3) that, with increasing doses, the developmental progression is retarded and application of the Kolmogorov and Smirnov two sample test (Gibbons, 1976) reveals this retardation as statistically significant with 10^{-7} moles RA ($P<0.01$), but not 10^{-8} or 10^{-9} moles of RA. The insets of Fig. 3 also show that with increasing doses of RA embryos become increasingly abnormal; all embryos treated with 10^{-7} moles are abnormal. Based on the number of morphological features (see Materials and Methods; Ghatpande *et al.*, 1991, 1993) in each embryo, the frequency distribution of embryos according to their rank was determined, which shows (Fig. 4) that, with increasing doses of RA, agreater proportion of embryos exhibit decreased number of morphological structures or rank values. The rank distributions for embryos treated with either dose of RA were compared with that for controls using the Kolmogorov and Smirnov test (Gibbons, 1976) and we find that the distributions are significantly different ($P<0.02$).

Graphic comparisons between the cell population size and blastoderm area for control and RA-treated embryos are shown in Fig. 5. Since each embryo has been separately identified prior to the analysis of different parameters, we have been able to designate the grade of abnormality to each data point on the graph. Control embryos appear as a relatively tight cluster (Fig. 5a) while with increasing doses of RA, embryos become heterogeneously distributed and grade I embryos appear as a cluster with the lowest cell

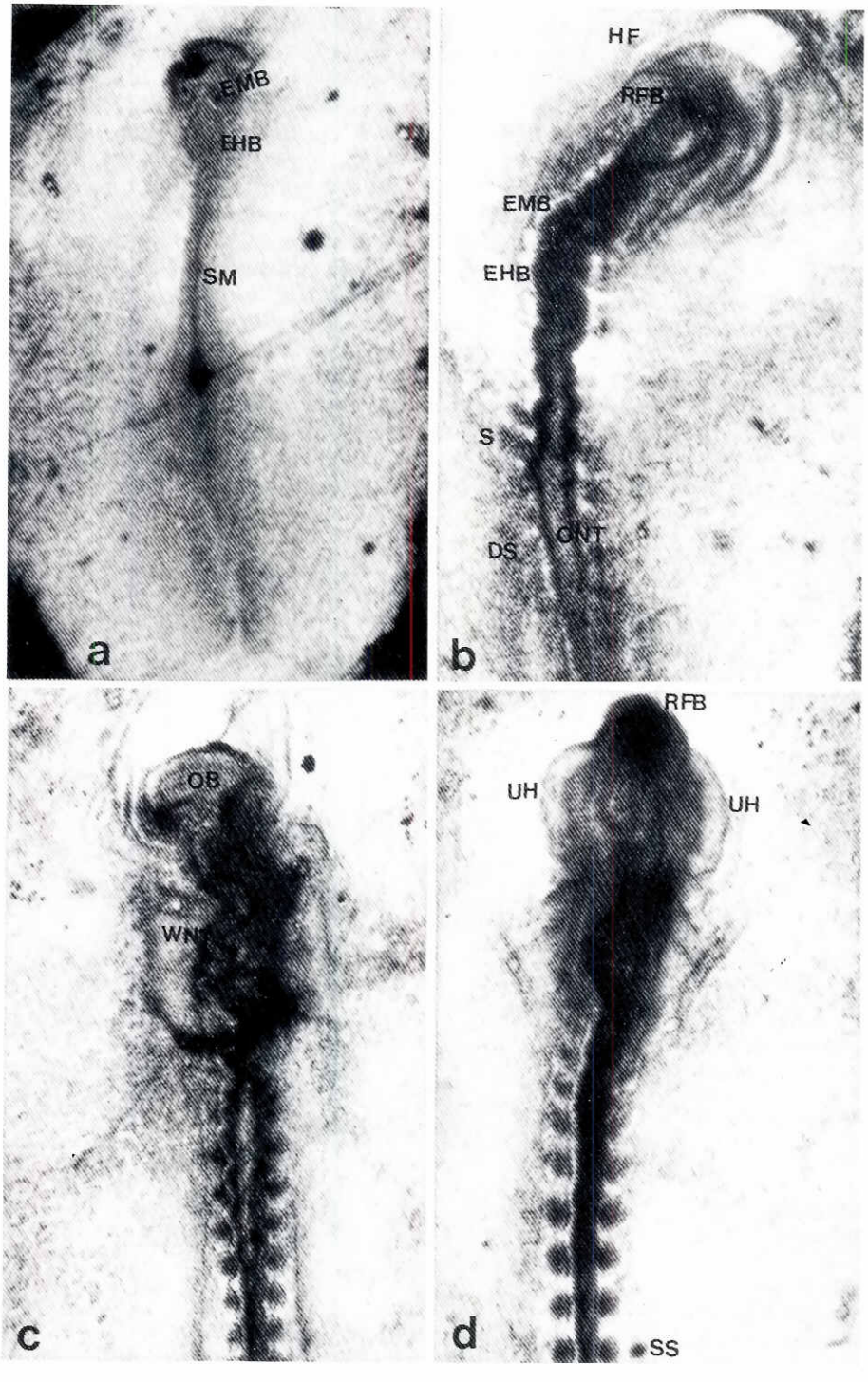

Fig. 2. (a) *Grade II embryo (rank= 11) without head fold and foregut, but with open brain with telencephalon missing and enlarged mid- and hindbrain. Open neural tube flanked by somite mesoblast (SM).* **(b)** *Grade II embryo (rank= 16) with head fold (HF), rudimentary forebrain (RFB), enlarged mid (EMB) and hind (EHB) brain, diffuse (DS) or winged somites (WS) and open neural tube (ONT).* **(c)** *Grade III embryo (rank= 27) with open brain (OB), wavy neural tube (WNT).* **(d)** *Grade III embryo (rank= 30) showing 2 unfused hearts (UH), reduced forebrain (RFB), enlarged mid- and hind-brain regions and a supernumerary somite (SS).*

number while embryos with grades II, III and IV also exhibit discrete clusters with increasing cell numbers (Fig. 5b-d).

Full primitive streak stage embryos contain $4.35x10^5 \pm 0.3x10^5$ cells (present data) and, from cell numbers after 20 h *in vitro*, the estimated mean cell population doubling time (T_G) in controls is 7.1±1.2 h, or with 2.8 doublings. In comparison, the T_G for all embryos treated with 10^{-7} moles of RA is the longest or 12.1±4.0 h (1.7 doublings), while with 10^{-8} and 10^{-9} moles of RA, the cell population doubling times decrease to 9.4±2.7 h (2.1 doublings) and 8.0±1.9 h (2.5 doublings), respectively. Among these, the T_Gs at two higher doses of RA are significantly longer than in controls ($P<0.05$) by Student's *t* test. Similarly, as compared to the mean doubling time for blastoderm area (T_A) of 10.9±1.7 h in controls, the estimated T_A for treated embryos are 14.6±5.6 h with 10^{-7} and 13.2±5.4 h with 10^{-8} moles of RA and both are significantly longer ($P<0.05$); T_A with 10^{-9} moles of RA is 10.8±3.9 h and not different from controls.

The relationship between the log cell number and developmental rank in shown in Fig. 6. Thus, with increasing doses of RA, the number of morphological structures decrease with a concomitant

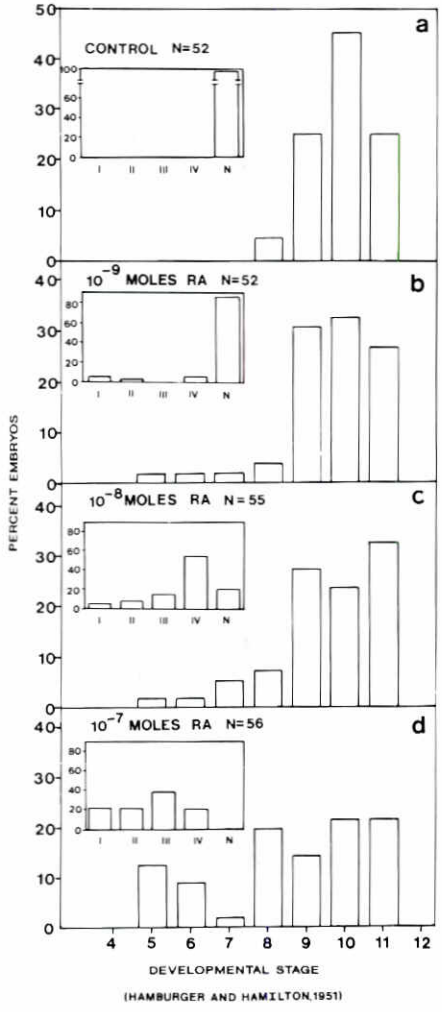

Fig. 3. Development of chick embryos treated with increasing doses of retinoic acid for 20 h *in vitro*. *Insets show the grade-wise distribution of abnormalities.* **(a)** *Control embryos,* **(b)** *treated with 10^{-9} moles of RA,* **(c)** *treated with 10^{-8} moles of RA,* **(d)** *treated with 10^{-7} moles of RA. Note the dose-dependent increase in abnormal embryos.*

reduction in the cell population size. We also observe that treated embryos distribute as discrete clusters with respect to the rank and cell number.

Finally, a comparison between the blastoderm area (mm^2) and rank (not shown) revealed that control embryos appear clustered with a mean area around 112±19 mm^2, while with increasing doses of RA, the distribution becomes heterogeneous.

Discussion

Primitive streak stage chick embryos treated with RA exhibit retardation of development (Figs. 3, 4), abnormal or unfused cardiac tubes with absence of heart in extreme cases, laterally extended or winged somites (Figs. 1, 2) similar to recent results (Osmond *et al.*, 1991; Sundin and Eichele, 1992). In grade I embryos, in the absence of neural tube, bilaterally positioned strips of somite primordia extend posteriorly but without somite differentiation. Fifty per cent of embryos treated with 10^{-7} moles of RA exhibit brain abnormalities and in 25% cases the anterior extremity of the forebrain (telencephalon) is either absent or severely reduced, while the mid- and hindbrain are enlarged and often open. This resembles the situation described for *Xenopus* embryos treated with RA (Durston *et al.*, 1989; Ruiz i Altaba and Jessell, 1991) which show deletion of anterior structures, and even a complete loss of head structures in most severe cases. Thus, RA induces caudalization in the chick embryo. We also find that increasing the dose of RA causes increasing severity of malformation (Figs. 3, 4) along with a reduction in the cell population size (Fig. 5) and increased duration of T_G (see Results). Thus, irrespective of their primary molecular targets or sites of action in metabolic pathways, RA (present data), aflatoxin B1 (Joshi and Joshi, 1981), isonicotinic acid hydrazide (Joshi *et al.*, 1990), trypan blue (Ghatpande *et al.*, 1991, 1993) and lithium chloride (Ghatpande *et al.*, 1993) inhibit cell population growth and perturb normal morphogenesis (Figs. 5 and 6). Our results are consistent with the specific

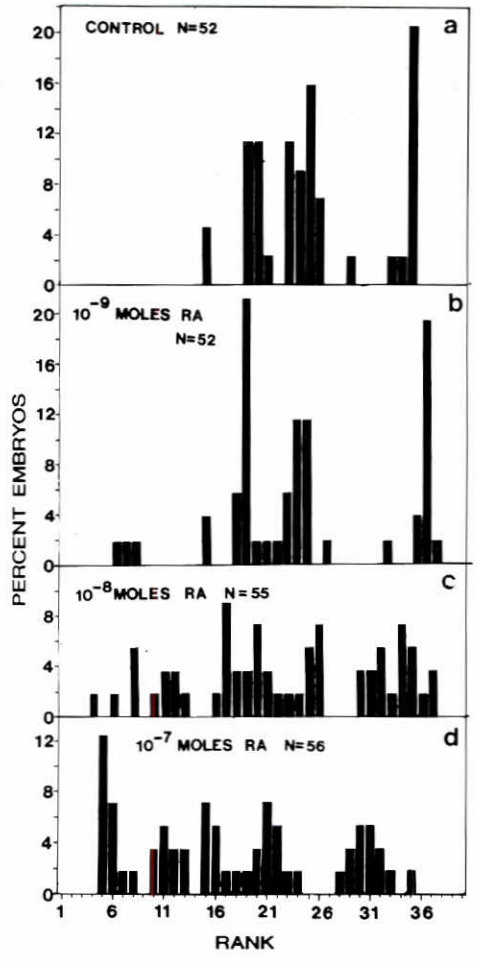

Fig. 4. Frequency distribution of embryos classified from the total number (rank) of morphological structures after treatment with increasing doses of RA. **(a)** *Control embryos,* **(b)** *treated with 10^{-9} moles of RA,* **(c)** *treated with 10^{-8} moles of RA,* **(d)** *treated with 10^{-7} moles of RA. The rank value distribution shifts towards lower values with increasing doses of RA.*

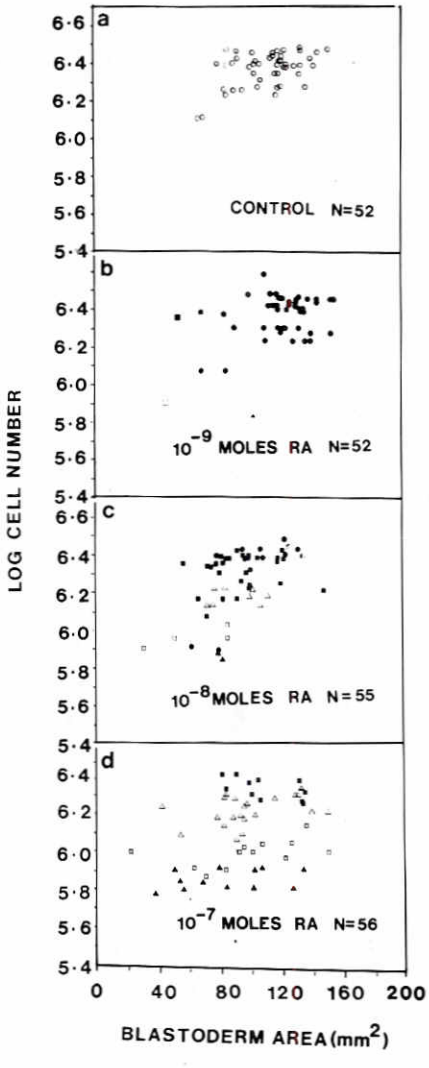

Fig. 5. The relationship between log cell number and blastoderm area of embryos. *As compared to the controls* **(a)**, *RA causes decreased cell population size and blastoderm area with* **(b)** 10^{-9}, **(c)** 10^{-8} *and* **(d)** 10^{-7} *moles of RA. (○) Control embryos; (●), treated normal embryos; (■) grade IV embryos; (▲) grade III embryos; (✱) grade II embryos; (Δ) grade I embryos.*

morphogenetic effects and associated inhibition of cell proliferation induced by RA during tail and limb regeneration and early development of the inner ear. Furthermore, our preliminary data (Rane and Modak, unpublished results) suggest that the cellular concentrations of transcripts for cyclins and cdc2 are also dramatically reduced in embryos treated with RA or LiCl.,

Examination of temporal changes in the relationships among parameters such as the blastoderm area, cell population size and development rank has revealed (Ghatpande *et al.*, 1993) that the latter two are independent parameters which control the expansion of blastoderm area. This also appears to hold for RA from the present data. Furthermore, similar to LiCl and trypan blue (Ghatpande *et al.*, 1993), RA also reduces the mean blastoderm area to 90-95 mm^2 as compared to 112 mm^2 in controls.

RA directly affects the differentiation of anterior dorsal mesoderm (Ruiz i Altaba, 1990), and the neural defects we observe may be caused at least partly by a perturbation of early mesodermal differentiation (Ruiz i Altaba and Jessell, 1991). A positive correla-

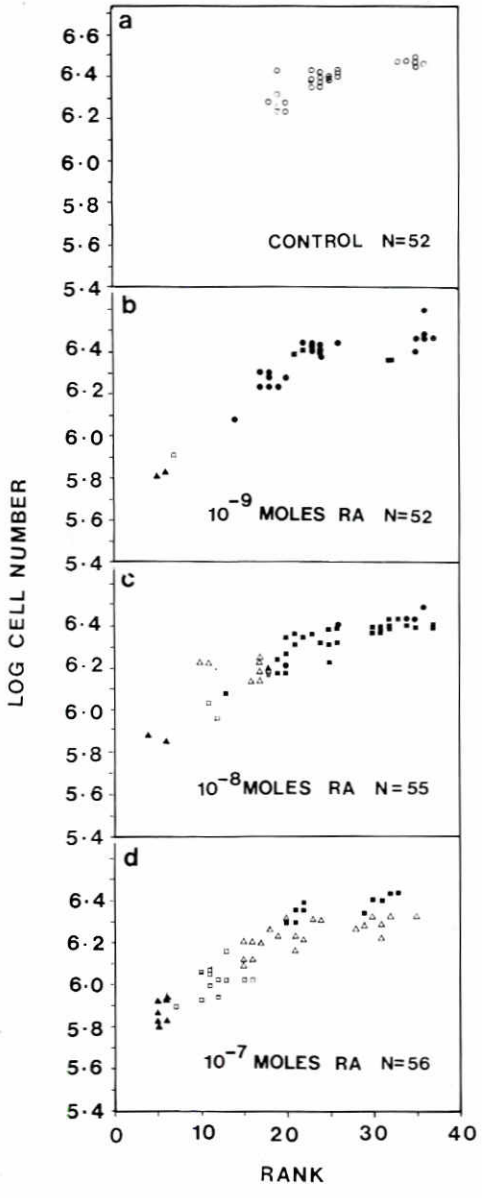

Fig. 6. The relationship between log cell number and morphological rank. (a) *Control embryos form 3 discrete clusters, while with treatment with* **(b)** 10^{-9}, **(c)** 10^{-8} *and* **(d)** 10^{-7} *moles of RA, increasing number of clusters with decreasing rank values are observed. As compared to data points for controls (○), RA-treated embryos were identified as treated normals (●) ad those exhibiting abnormalities graded as (■) IV, (Δ) III, (□) II and (▲) I.*

tion between high expression of cellular RA-binding proteins in tissues which are most affected in retinoic acid embryopathy has also been reported (Balling, 1991). Recently, Sundin and Eichele (1992) have shown that RA induces ectopic expression of Ghox 2.9 gene in chick embryos in anterior ectoderm of the late gastrula followed by abnormal development of mid- and hindbrain. In *Xenopus*, RA suppresses archencephalic structures (Durston *et al.*, 1989) concomitant to an increased expression of X1Hbox-6 (Wright *et al.*, 1990), a neural marker of trunk-tail level. Similarly, RA suppressed the expression of XCG-1, XAG-1 and XA-1 which are markers for anterior structures, and simultaneously enhanced the expression of deuterencephalic marker genes XIF-3 and X1Hbox-6 (Sive *et al.*, 1990). LiCl also inhibits the formation of anterior/archencephalic structures in early embryos of chick (Nicolet, 1965) and *Triturus phyrrogaster* (Masui, 1958) and causes caudalization (Masui, 1958). LiCl (Nicolet, 1965; Ghatpande *et al.*, 1993), trypan blue (Ghatpande *et al.*, 1991, 1993) and RA (present data) suppress cell population growth and cell cycle-specific transcripts (Rane and Modak, unpublished results) with increasing severity of malformation in the chick. Therefore, we suggest that the expression of early pattern-forming genes may be causally related to positionally defined cell proliferative activity, and any perturbation in the latter may lead to a modification of the developmental pattern or vice versa. RA, suggested to be a natural morphogen (Tickle *et al.*, 1982; Thaller and Eichele, 1987; Green, 1990), is synthesized at high concentration in the Hensen's node, which is the center of gastrulation (Nicolet, 1971). Therefore, the present data, and that on LiCl and trypan blue (Ghatpande *et al.*, 1993) are consisted with the suggestion (Yamada, 1990 and 1993, unpublished) that caudalization may involve a negative cell cycle control. We further suggest that a negative control of cell cycle may be linked to the suppression of anterior homeobox gene expression and a promotion of expression of homeobox genes responsible for caudalization. This, however, needs to be verified by examining cell cycle behavior in different organ primordia.

Materials and Methods

Embryo culture

Fresh fertilized White Leghorn eggs were incubated at 38°C, and full primitive streak stage 4 (Hamburger and Hamilton, 1951) embryos were cultured *in vitro* (New, 1955) with 1.0 ml thin albumen as the nutrient. Retinoic acid (retinol, all-trans, Sigma, USA) was dissolved in DMSO to obtain a 0.1 M stock solution and aliquots were stored at -20°C. For treatment, stock RA was diluted in Pannett-Compton (PC) saline to obtain 10^{-3} M, 10^{-4} M and 10^{-5} M. 100 µl RA solution (10^{-7}, 10^{-8} or 10^{-9} moles) was pipetted over the blastoderm, allowed to diffuse (30 min, 25°C) and embryos were incubated for 20 h at 38°C. A few embryos were sham-treated with 0.0001%. DMSO had no effect on development (data not shown).

Estimation of blastoderm area and morphological rank

Along with 52 controls, 162 embryos were treated with RA. Blastoderm outlines were traced (x67) on a camera lucida at the beginning (T_0) and after 20 h, and blastoderm area was estimated by planimetry (Ghatpande *et al.*, 1991). After culture, developmental stages (Hamburger and Hamilton, 1951) reached by controls and equivalent stages of RA-treated embryos were determined. All morphological structures were noted to estimate the embryo rank (Ghatpande *et al.*, 1991, 1993). This method allows a numerical classification of embryos based on the number of morphological structures irrespective of the extent of normal or abnormal development. RA-treated embryos were examined for the grade of abnormality as before (Rao and Chauhan, 1971).

Estimation of cell population size

In each experiment, untreated control embryos of the same stage as treated embryos of the same abnormal grade were pooled, suspended in 10 vol of PC and homogenized. Nuclei were stained with ethidium bromide (0.2 mg/ml, 20 min, on ice) and counted in a Neubauer's chamber under epifluorescence (Polyvar II, Reichert) to estimate the total nuclei as before (Ghatpande *et al.*, 1990).

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