Chick Limb Duplications Produced by Retinoic Acid Releasing Microimplants

Lisa B. Nass, Annette J. Schlueter and Grayson S. Davis
Department of Biology
Valparaiso University
Valparaiso, Indiana 46383

Introduction

Several recent studies have suggested that the limb bud vasculature may act in determining the skeletal pattern of the limb (3) by establishing metabolic gradients which would control the local differentiation of muscle or cartilage (1). Furthermore, systemic application of vitamin A, or its acid, retinoic acid, to developing embryos has been shown to produce both skeletal malformation and abnormal vascularization of the limb (7, 4). When microimplants of filter paper containing retinoic acid are implanted into developing limb buds, they can induce duplications of the limb skeleton (11), apparently mimicking the action of the polarizing region. Our initial interest was to observe the effect upon the vasculature produced by retinoic acid implants. However, we were unable to reliably produce duplications with filter paper implants. An alternative implant, an ion exchange bead, was suggested by Bruce M. Alberts, Department of Biochemistry and Biophysics, University of California, San Francisco (2). A comparison of these two carriers revealed that the ion exchange implanting method was far more reliable, less toxic, and less likely to induce other malformations than the filter paper implanting method.

Methods

For the paper implants, Rhode Island Red chick eggs were incubated under standard conditions for three days to stages 18 to 20 (6) while being turned twice daily. The eggs were then windowed as described by Hamburger (5) except that the windows were broken into the shell with forceps rather than sawn with a hacksaw blade. This method is reliable for early stage chicks and much faster than sawing. The amniotic fold directly over the right wing bud was pulled back using an electrolytically sharpened tungsten wire probe and a slit was then made into the anterior portion of this bud using the same probe.

A small piece (0.5mm x 0.5mm) of Whatman diethylaminoethyl cellulose (DEAE) filter paper was prepared for implanation by being soaked for one minute in one of a series of concentrations of all trans-retinoic acid (Sigma, type XX) dissolved in dimethyl sulfoxide (DMSO; Sigma grade 1). The solutions for each experiment were made from a freshly opened ampoule of retinoic acid and kept in darkness to minimize decomposition of the retinoic acid. Paper soaked longer than one minute tended to disintegrate when implantation into the slit was attempted. This paper was then implanted into the slit such that the paper extended through the apical ectodermal ridge and into the limb mesoderm adjacent to somites 15, 16 and 17. A free edge of the paper remained outside the limb bud. The window was then sealed with cellophane tape and the egg reincubated.

After seven days, the embryo (now at stage 35 to 37) was removed from the egg, rinsed in physiological saline and fixed in Bouin's fixative. The fixed embryo was stained with 1.2% solution of Victoria Blue B dye (Sigma) to stain the cartilages, dehydrated in a graded ethanol series (50%, 70% and 95%) and transferred to methyl salicylate to clear the flesh so that the skeletal elements could be examined for duplications.

For the bead implants, the same method was used up to and including slitting the wing bud with the tungsten probe. However, instead of introducing the retinoic acid in filter paper carriers, AG1-X2 ion exchange beads (Formate form, 100-200 mesh, Bio-Rad Laboratories) were used. These beads, made of a styrenedivinylbenzene crosslinked lattice with attached quaternary ammonium groups, exchanged electrostatically bound formate ions for retinoic acid ions when soaked for 20 minutes in one of a series of concentrations of retinoic acid in DMSO (2). The beads loaded with retinoic acid were then rinsed twice with ten minute changes of Hank's balanced salt solution and implanted. The implanted bead was completely surrounded by the

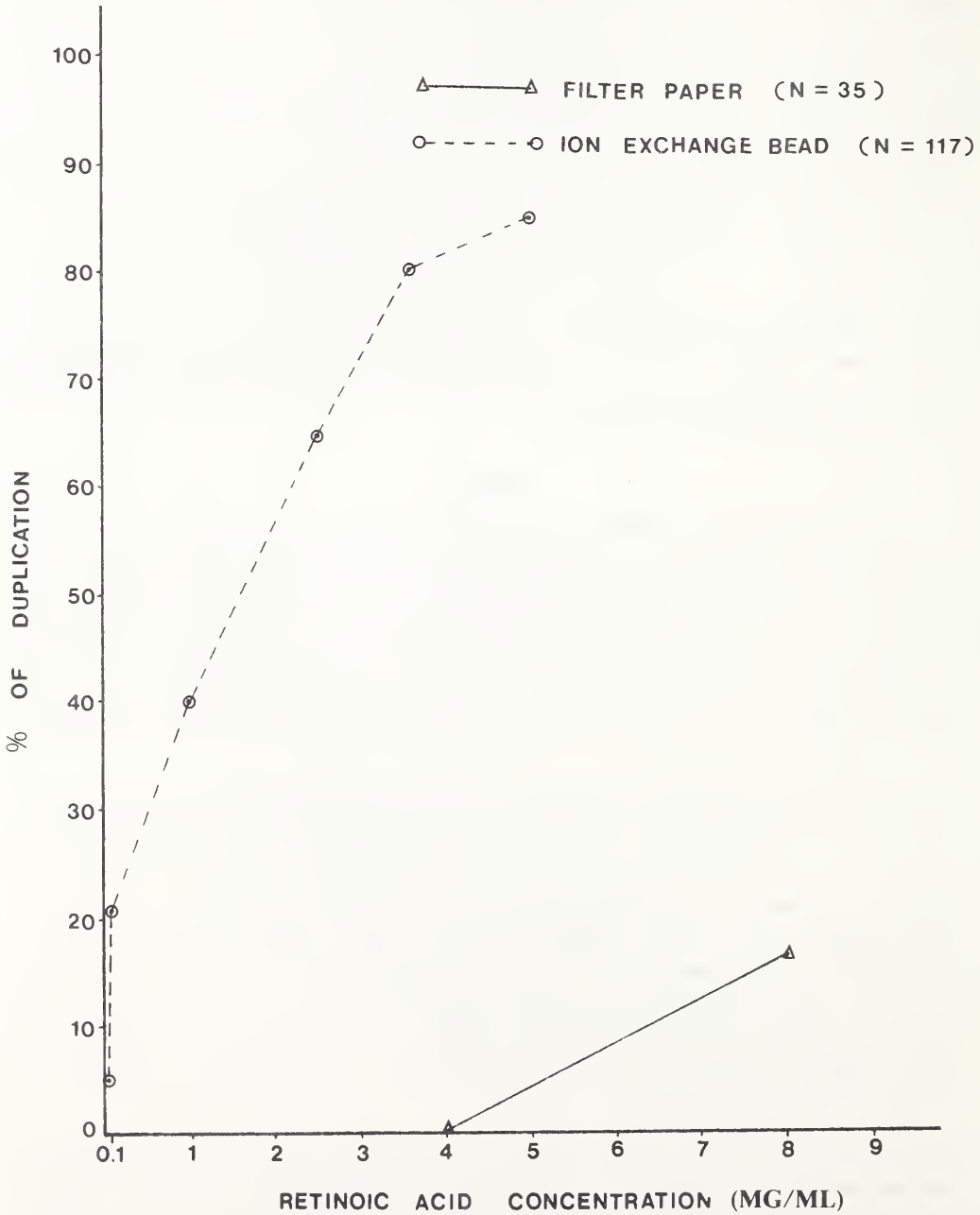

FIGURE 1. Dose response curves for filter paper and ion exchange bead implants: percent of surviving embryos with limb duplications versus retinoic acid concentration.

mesoderm just beneath the apical ectodermal ridge. The egg was next sealed with cellophane tape and reincubated. The embryo was later fixed, stained and cleared as before. Controls were run for both implanting processes using either filter paper or beads soaked in DMSO alone. Several bead-implanted and several paper-implanted embryos were fixed after only one or two days of incubation and examined to see if the implants had slipped out of the limb.

Results

All control embryos examined one or two days after implantation of either filter paper or beads still retained their implants. Furthermore, once 10 day embryos were cleared, it was often possible to find the implant still in the limb. When loaded with retinoic acid, both types of implant were capable of producing duplications in the cartilages of the autopod. Bead implants produced duplications when loaded with lower concentrations of retinoic acid than did the paper implants (Figure 1). Trying to produce more duplications by increasing the retinoic acid concentration loaded into the paper produced an increase in the death rate to a value much greater than that obtained with the bead implants (Figure 2). Moreover, nearly 100% of the embryos surviving

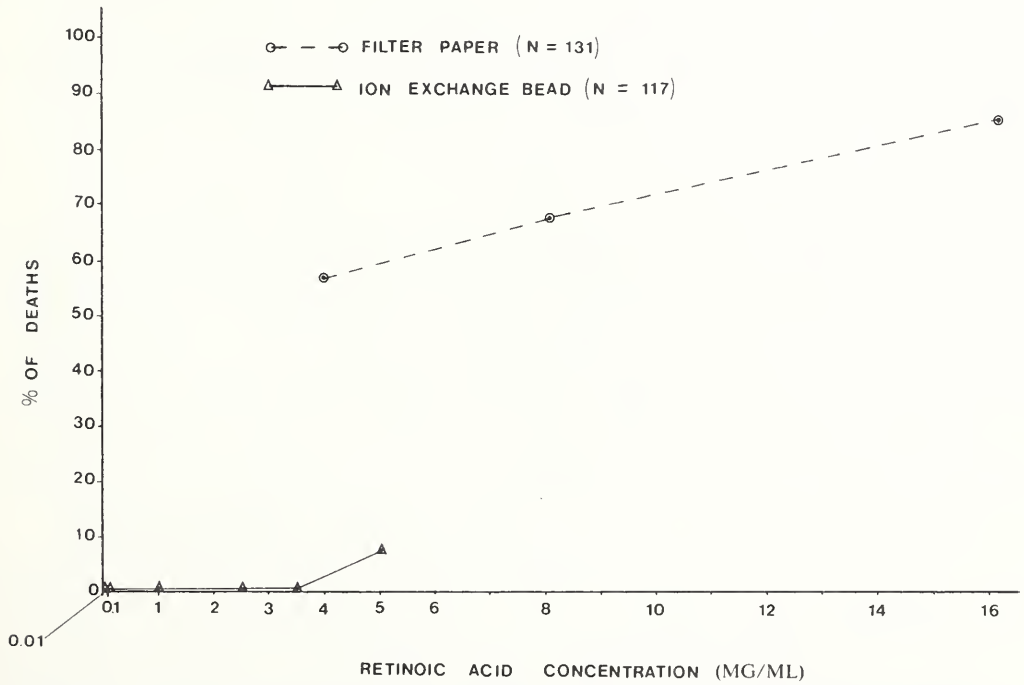

FIGURE 2. Dose response curves for filter paper and ion exchange bead implants: percent of deaths unattributable to contamination or injury at the time of implant versus retinoic acid concentration.

the paper implant technique were malformed. These embryos developed brain deformations, beak deformations, ectopia cordis (heart exterior to the chest cavity), or extensive abdominal herniation of the gut. Such abnormalities were common but not universal in control embryos implanted with filter paper soaked in DMSO alone. Only one of the 117 surviving bead-implanted embryos showed a detectable malformation.

When the results of the 5mg/ml bead-implanted embryos were examined, substantial differences in the length of the duplicated digits were noted. Embryos implanted at earlier stages had longer duplications (Figure 3).

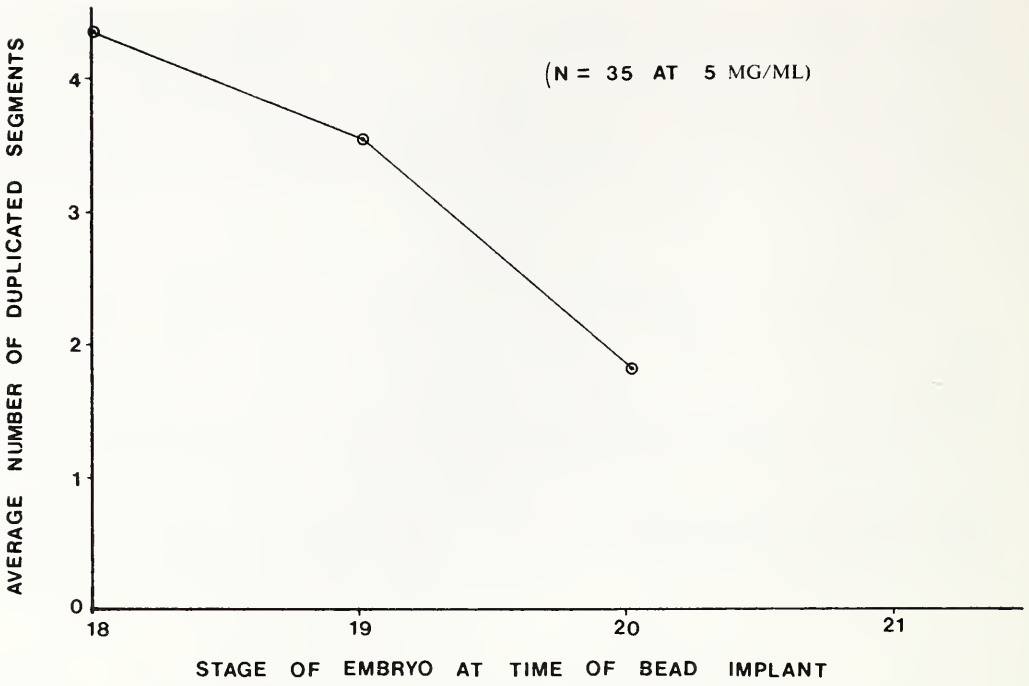

FIGURE 3. Average number of duplicated segments versus the stage of the embryo at the time the bead was implanted.

Discussion

The data from the two types of carriers showed significant differences. A much higher rate of duplication was obtained with lower retinoic acid concentrations when using ion exchange beads as opposed to filter paper. Specifically, an 83% duplication rate was obtained at a concentration of 5mg/ml of retinoic acid using ion exchange beads, significantly better than a 17% duplication rate at a concentration of 8mg/ml using the filter paper carrier (see Figure 1). This may be attributable to the fact that the bead releases retinoic acid in lower concentrations and over a longer period of time than does the paper (2). Since neither paper nor bead shows an inclination to slip out of the limb after proper implantation, we cannot attribute the lower percent duplication obtained with paper to the failure of paper to remain implanted.

At the same time, the death rate (unattributable to contamination or embryonic injury at the time of implantation) was much lower for bead-implanted embryos than for filter paper-implanted embryos. At a 5 mg/ml concentration of retinoic acid using the ion exchange bead method, a death rate of only 7% was observed, whereas at a 4 mg/ml concentration of retinoic acid using the filter paper method a 56% death rate resulted (Figure 2). Because the malformations in the filter paper-implanted embryos occurred in the controls as well as the experimental chicks, it may be that this effect was due to the filter paper itself, or, more likely, to the relatively large amount of DMSO each filter paper implant carried. Furthermore, the embryos treated with retinoic acid-containing paper implants almost universally developed with head deformations, heart exterior to the chest cavity or gut exterior to the abdominal cavity. In contrast, the ion exchange bead treated embryos showed only one case of deformation (at a concentration of 5 mg/ml).

The fact that implanting young embryos produced longer (proximal to distal) duplications is not surprising given the popular model of sequential proximal-distal specification of limb pattern (8). According to this model, increasingly shorter and more distal regions of the limb would be labile to alterations, including duplications,

of pattern at later stages (9). We were surprised that a stage 18 implant would produce duplications in the autopod alone. Experiments in which limb development is interrupted by removal of the apical ectodermal ridge indicate that at stage 18 pattern specification is not yet effectively complete for the zeugopod or even the most distal portion of the stylopod (8, 10).

We are convinced that retinoic acid implants are an effective tool for producing duplications in the pattern of limb cartilages. Of the two carriers we compared, the ion exchange beads are by far the more reliable and less damaging to the embryo. We are now examining the early effects of retinoic acid implants upon the vasculature of the limb bud.

Literature Cited

1. Caplan, A.I., and S. Koutroupas, 1973. The control of muscle and cartilage development in the chick limb: the role of differential vascularization. J. Embryol. exp. Morph. 29:571-583.
2. Eichele, G., C. Tickle, and B.M. Alberts. Micro-controlled release of biologically active compounds in chick embryos: beads of 200 um diameter for the local release of retinoids. In preparation.
3. Feinberg, R.N. and J.W. Saunders, Jr., 1982. Effects of excising the apical ectodermal ridge on the development of the marginal vasculature of the wing bud in the chick embryo. J. exp. Zool. 219:345-354.
4. Fraser, B.A., and A.A. Travill, 1978. The relation of aberrant vasculogenesis to skeletal malformation in the hamster fetus. Anat. Embryol. 154:111-120.
5. Hamburger, V., 1942. A Manual of Experimental Embryology. University of Chicago Press, Chicago.
6. Hamburger, V., and H. Hamilton, 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49-92.
7. Kochhar, D.M., 1977. Cellular basis of congenital limb deformity induced in mice by vitamin A. Proceedings of the Second International Conference on Morphogenesis and Malformation. Birth Defects: Original Article Series 13:111-154.
8. Saunders, J.W. Jr., 1948. The proximo-distal sequence of the origin of the parts of the chick wing and the role of the ectoderm. J. exp. Zool. 108:363-404.
9. ____, M.T. Gesseling, and J. Errick, 1976. Inductive activity and enduring cellular constitution of a supernumerary apical ectodermal ridge grafted to the limb bud of the chick embryo. Devl. Biol. 50:16-25.
10. Summerbell, D., 1974. A quantitative analysis of the effect of the excision of the AER from the chick limb-bud. J. Embryol. exp. Morph. 32:651-660.
11. Tickle, C., B. Alberts, L. Wolpert, and J. Lee, 1982. Local application of retinoic acid to the limb bud mimics the action of the polarising region. Nature, Lond. 296:564-565.